CURRENT LABORATORY PROCEDURES

BOARD OF HEALTH TERRITORY OF HAWAII

By
BERNARD WITLIN

C. L. WILBAR, JR., M. D.
President, Board of Health
Territory of Hawaii

FOREWORD

The growing demand for more rapid and accurate laboratory work throughout the Territory of Hawaii has made it desirable that satisfactory and standardized methods be employed, so that results obtained by a laboratory in one locality may be comparable with the results in the other laboratories throughout the Territory.

This manual has been divided into six sections as a matter of convenience for the physician. The first section is arranged in a table listing the names of the diseases, specimens desired, examinations made and materials supplied by the Board of Health, Territory of Hawaii. Symbols beneath the names of the individual diseases refer to whether or not the diseases are reportable, isolationable or quarantinable and the duration of the time from which food handling is to be abstained.

Section II consists of simple directions for the collection of specimens requiring special technique and/or precaution when being submitted to the laboratory.

Section III is a concise outline of disease carriers and prescribed precautionary measures for such carriers.

Section IV is a copy of the United States Postal Laws and Regulations governing the transportation and shipping of laboratory specimens by mail.

Section V gives the procedures employed by the laboratories of the Board of Health, Territory of Hawaii. These are strictly current procedures based on actual use and no claim is made for superiority of any of the procedures over many others in use. They have been carefully tested in our laboratories and found satisfactory for our purposes.

Section VI is a compilation of culture media, stains and reagents referred to throughout Section V. Culture media, stains and reagents have been given reference numbers (i.e., blood agar[4], Obermayer's reagent[49]). All numbers refer to the numbers assigned each medium or reagent as listed in the last section of the manual (Culture media and Reagents).

In the preparation of this manual, the educational qualifications of persons performing the work are taken for granted and little other than laboratory procedures in "cook book" fashion is presented. It is assumed that persons desiring more information on any of the diseases concerned will consult standard texts for supplementary information.

BERNARD WITLIN, Sc. D.
Bacteriologist
Territory of Hawaii

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I. Diseases Reportable in the Territory of Hawaii

LABORATORY SERVICE OFFERED PHYSICIANS BY BOARD OF HEALTH

CLINICAL SPECIMENS FOR LABORATORY EXAMINATION

Disease	Specimen	Examination	Supplied By Board of Health
Actinomycosis *	Pus from abscesses	Stained slide for actinomyces Culture, isolation and identification of A. bovis and A. actinomycetem—comitous	Sterile containers Sterile swabs
Amebiasis *	Warm feces Feces in preservative	Saline and stained preparation for trophozoites and cysts Examination for cysts Occult blood	Stool containers Mailing containers Preservative
Anaerobic Infections	Pus	Anaerobic culture Animal inoculation	Sterile swabs Sterile test tubes
Anthrax *I. (Pneumonic)	Swab of skin pustule, blood, citrated, sputum, tissue (hair) hides, brushes, etc.	Stained slide for B. anthracis Culture, isolation and identification of organisms Animal inoculation	Sterile containers
Bacillary Dysentery * I. F.H. (until negative)	Feces Feces in preservative	Culture, isolation and identification of organisms Occult blood	Stool containers Mailing containers Preservative
Botulism	Suspected food	Culture, isolation and identification of organisms Animal inoculation	Sterile containers
Chancroid *	Pus or gland juice from buboes before suppuration	Stained slide for H. ducrey Culture for isolation and identification of organisms	Slide mailing containers Sterile tubes of inactivated rabbit blood for culturing Ducrey's bacillus
Cholera * I. Q. F.H. (until neg.)	Feces Vomitus	Direct microscopic examination of wet smear for V. cholera Culture, isolation and identification of organisms	Sterile containers

*—Notifiable
I.—Isolation of Patient
Q.—Quarantinable
F.H. (followed by length of time)—Prevention from practicing food handling

CLINICAL SPECIMENS FOR LABORATORY EXAMINATION

Disease	Specimen	Examination	Supplied By Board of Health
Diphtheria and Carrier State * I. Q. F.H. (until neg.)	Swab	Stained slide for C. diphtheriae Culture Virulence test	Sterile swabs Loeffler's media
Filariasis *	Blood, film Blood, citrated Chylous urine	Stained smear	Slide Mailing containers
Food Poisoning	Suspected food Feces Vomitus	Culture, isolation and identification of organisms Animal inoculation Occult blood	Sterile containers Mailing containers
Fungus Infection	Pus Skin scrapings	Smears Cultures	Sterile test tubes Mailing containers
Gonorrheal Infections * I. F.H. (until cured)	Slide preparation Material (exudate) for culture	Stained for gram negative intracellular diplococci Culture, isolation of organisms	Slide mailing containers Sterile swabs Sterile broth for swabs Mailing containers
Infectious Mononucleosis	Blood, clotted	Heterophile test	Sterile test tubes
Intestinal Parasites F.H.	Feces Anal swabs (for enterobius ova)	Wet preparation Stained preparation Occult blood	Stool containers Mailing containers
Leprosy * I. (Leprosarium)	Smears Snips Scrapings Biopsy tissue	Stained slide for Hansen's bacillus	Specimen containers Slide mailing containers
Malaria * I.	Blood film	Stained for parasites	Slide mailing containers
Meningitis* **a–Meningococcic** * I. Q. **b–Influenzal** **c–Pneumococcic** **d–Staphylococcic** **e–Streptococcic** **f–Other**	Spinal fluid Spinal fluid Nasopharynx swab	Microscopic examination of culture for isolation of organism Agglutination Globulin reaction Colloidal mastic test Qualitative sugar Culture for determination of carrier state	Sterile test tubes, cork stoppered Sterile swabs

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CLINICAL SPECIMENS FOR LABORATORY EXAMINATION

Disease	Specimen	Examination	Supplied By Board of Health
Paratyphoid Fever (A and/or B) * I. Q. F.H. (1 yr. neg.)	Blood, clotted Blood, citrated Feces Feces, preserved Urine	Agglutination test Culture, isolation and identification of organisms	Sterile test tubes Stool containers Stool containers with preservative Mailing containers Kracke medium for blood culture
Pertussis * I.	Spray from cough on cough plate Blood, citrated	Culture for H. pertussis Agglutination test	Cough plates Sterile test tubes Sterile swabs
Plague * I. Q. **Bubonic Septicemic Pneumonic**	Fluid aspirated from buboes Blood Sputum	Stained smear for B. pestis Culture Animal inoculation (Not to be shipped. Notify local health officer for proper procedure)	Slide mailing containers Sterile containers
Pneumonia (Pneumococcic and other types)	Blood, citrated Sputum Pleural fluid Spinal fluid	Culture Type determinations Quellung reaction Mouse inoculation	Sterile containers Mailing containers
Poliomyelitis * I. Q. F.H. (2 weeks)	Spinal fluid	Microscopic examination Qualitative sugar Globulin reaction Colloidal mastic	Sterile containers Mailing containers
Q. Fever	Blood, clotted (3 weeks to 2 months after recovery)	Complement fixation test	Sterile test tubes
Rat Bite Fever	Lesion exudate Blood, citrated taken at height of febrile paroxysm	Darkfield Animal inoculation	Sterile citrated test tubes
Septic Sore Throat * F.H. (2 wks.)	Throat swabs	Culture for hemolytic strep	Sterile swabs
Septicemia	Blood, citrated	Stained slide for organisms Culture and identification	Kracke-citrate media Sterile and citrated test tubes

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CLINICAL SPECIMENS FOR LABORATORY EXAMINATION

Disease	Specimen	Examination	Supplied By Board of Health
Syphilis * I. F.H. (until under treatment)	Blood, clotted Spinal fluid Exudate from lesion	Kahn precipitation Kolmer complement fixation Kline, Eagle flocculation Globulin Colloidal mastic Darkfield	Sterile test tubes Darkfield collection set
Trichomoniasis (Trichomonas vaginalis)	Vaginal smear Freshly voided urine	Microscopic hanging slide	Sterile swabs Containers
Trichinosis *	Biopsy materials	Pepsin digestion followed by examination for Trichinella spiralis	Specimen containers Mailing containers
Tuberculosis, Pulmonary * I. F.H. (until inactive)	Sputum Urine Pleural fluid Spinal fluid Gastric lavage	Stained slide for acid-fast bacilli Digestion & culture of organisms Animal inoculation	Sterile sputum containers Sterile test tubes Mailing containers
Tularemia *	Blood, clotted	Agglutination test	Sterile test tubes
Typhoid Fever and Carrier State * I. Q. F.H. (1 yr. neg.)	Blood, clotted Blood, citrated Feces Bile Urine	Agglutination test (Widal) Culture, isolation and identification of organisms Occult blood	Sterile test tubes Stool containers Mailing containers Kracke medium for blood cultures
Typhus Fever *	Blood, clotted	Agglutination test (Weil-Felix)	Sterile test tubes Mailing containers
Undulant Fever	Blood, clotted Blood, citrated	Agglutination test (Huddleson) Culture	Sterile test tubes Sterile citrated test tubes
Vincent's Infection	Slide preparation	Stained for organisms of Vincent	Slide mailing containers
Weil's Disease *	Blood, clotted Blood, citrated Urine	Agglutination test Animal inoculation Darkfield	Sterile test tubes Sterile citrated test tubes Sterile urine bottles Mailing containers

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II. Method for the Collection of Specimens

The following is a list of the techniques to be employed when material is submitted to the laboratory for examination.

AMEBIASIS

It is preferable to have the patient pass his stool at the place of examination so that the examination may be made without delay, or if this is not possible, that the stool be kept at body temperature until it is examined (place container with stool in jar of water at body temperature). An increased number of motile forms may be obtained by administering a saline cathartic (castor oil and mineral oils should be avoided as they complicate the microscopic picture). The feces should be collected in a clean, dry and preferably sterile container.

Vegetative forms of Endamoeba histolytica degenerate soon after specimens are collected. Stools containing encysted amoebae may be satisfactorily preserved by the addition of five per cent formalin.

Films prepared from ulcers in the intestinal mucosa or from freshly collected stools should be fixed in Schaudinn's fluid (60° C.—ten minutes or overnight at room temperature) and then kept in 70 per cent alcohol until the examination can be completed.

Specimens may also be collected through a proctoscope from ulcerated areas in the intestinal mucosa.

At least six successive daily examinations should be carried out before a patient is considered free from infection.

DIPHTHERIA

1. Two sterile swabs should be used for each person cultured—one to obtain material from the throat lesion or tonsillar crypts, the other to be passed to the nasopharynx through one nostril. For diagnostic purposes the swabs should be kept apart.

2. Swabs, labeled "nose" and "throat," should be brought into the laboratory as soon as possible after they have been taken. Long delays in transporting swabs to laboratory are believed to cause a considerable (but unknown) reduction in numbers of viable organisms, especially on swabs from convalescent and healthy carriers. The preferable time limit for bringing the swabs into the laboratory is two hours.

Swabs should be kept as warm as possible. Body temperature is preferred, but room temperature will suffice.

3. The laboratory supplies sterile swabs in sterile tubes.

GONORRHEA

A. Preparation of material—Glass slides and swabs

1. The glass slides used should be chemically clean and free from grease. The method recommended by the laboratory to obtain a clean, greaseless slide is to clean glass slides with Bon Ami.
2. Sterile swabs, of the same general type as used for nose and throat swabbing, are supplied by the laboratory.

B. Method of taking smear, fixing smear and wrapping for transit to laboratory

1. To prepare a smear, the exudate which has been obtained on a sterile swab is "rolled out" over the surface of two glass slides by rotating the applicator between the thumb and index finger. The "rolling" procedure is superior to rubbing the swab over the glass surface. By this method the pus cells remain intact and more gonococci retain their intracellular position. The smears or "spreads" should be so placed nearer one end of the slide that space remains available at the opposite end for labeling. The success of the examination depends directly upon the care with which the smear is made. Thick films are unsatisfactory and can be avoided by collecting only a small amount of exudate and by rolling the swab over the surface but once. A well-made preparation should be not more than one cell thick. When the slide has been air dried and the film fixed by gentle heating of the reverse side of the slide, it should be marked plainly with the patient's name, the source of the specimen and the date.
2. Place toothpicks or pieces of wooden applicators at either end of the slide, place another slide on top of smear and place two elastic bands around either end. This insures cleanliness and eliminates the possibility of injuring the smear in transit. The Board of Health supplies individual and double slide mailing containers upon request.

C. Collection of specimen

1. Male urethra

   Aseptic technic is, as a rule, unnecessary in obtaining cultures of inflammatory exudates. If such precautions seem advisable, the end of the penis is cleansed with soap and water and a mild disinfectant, such as 1:40,000 solution of mercury oxycyanide applied. The specimen of pus at the meatus is taken by the use of a sterile cotton swab. If the amount is insufficient, the penile urethra should be stripped. The swab should be placed immediately in a test tube containing 0.15 c.c. of meat infusion broth or any other menstruum, such as sterile ascitic fluid, that is not injurious to the gonococcus. More difficulty is encountered in obtaining specimens from chronic cases of prostatitis and posterior urethritis. Prostatic secretions can be obtained by compressing the anterior urethra with the thumb and finger, meanwhile massaging the prostate with the other hand. The constriction of the urethra is then released, and the exudate which has accumulated in the urethra is collected in a dry, sterile test tube, or, if the quantity is scant, in about 0.15 c.c. of broth. The first few drops of urine will contain any exudate from the posterior urethra, and should be collected in about 0.15 c.c. of broth. Urine, for culture, may be voided directly into a sterile test tube.

2. Female genitalia

   Sterile preparation of the vulva and douching of the vagina are seldom necessary. The urethral meatus is cleansed by a sterile cotton pledget and the urethral exudate then collected on sterile swabs. It is important to apply pressure to Skene's ducts and to the urethra to obtain sufficient material for a satisfactory examination. Cultures from the cervix and vagina should be made with the aid of a sterile speculum, used without lubricant unless it is absolutely necessary. The cervix should be compressed with the blades in order to express glandular secretions in the deeper tissues, and the exudate is collected on a sterile swab. King Mascall emphasizes the taking of a specimen from behind the cervix, in the region of the posterior fornix, especially if the question of cure is involved. Care should be exercised to

obtain a sufficient quantity of exudate, since successful inoculation is dependent upon this. In taking cultures from infants and children, it is important that the swab be inserted into the vagina, and not, as so frequently happens, brought merely in contact with the vulva. An ear speculum is useful for this purpose.

3. Miscellaneous
   In taking specimens from the ano rectal region, aseptic technic is important. Sterile swabs are used for collecting the material, after thorough preparation of the area with soap and water followed by suitable antiseptics.
   Pus from the conjunctiva and from abscesses is suspended in infusion broth. Joint and spinal fluids and urine are collected in sterile tubes without broth. For blood cultures, about 10 c.c. of blood is obtained by venipuncture and 1 c.c. and 4 c.c. amounts immediately placed in flasks containing 100 c.c. of glucose ascitic fluid broth; the remaining 5 c.c. is added to from 2 to 4 c.c. of 2.5 per cent sodium citrate in order to prevent coagulation, and is made into blood agar plates in the laboratory.

D. Precautions in handling exudates before culturing

1. The most important precaution is to prevent the exudate from drying on the swab before suspending it in broth or ascitic fluid. Drying is accompanied by rapid death of the gonococci.
   It is generally believed that exudates to be cultured for gonococci should be kept warm from the time they are taken until cultures have been made; however, low temperatures (from 4° to 10° C.) have only a slightly injurious effect on broth suspensions of gonococcal pus, even after 6 or 8 hours. When suspensions of purulent exudate are permitted to remain at room or incubator temperature for a few hours, the contaminating organisms present usually overgrow the gonococcus, making the isolation of the organism difficult. Broth suspensions of gonococcal pus should be kept cool or at refrigerator temperature, rather than at room or incubator temperature, until they can be cultured. Best results are obtained when cultures are made immediately after the specimen is collected.

LEPROSY

A. Macular and nodular specimens

1. Microscopical preparations should be made from the macules, papules or nodules which may be present on the skin.
2. Thoroughly scrub skin with soap and water followed by alcohol and ether, to free skin from any saprophytic acid-fast micro-organisms that might be present on the surface. Grasp lesion between thumb and index finger and apply pressure until overlying skin becomes anaemic. With a sterile scalpel make a small incision through the epidermis and well into the corium. Also lightly scrape the surface of the skin. This causes a small amount of serous exudate which is spread upon microscope slides. The obtaining of specimens with large amounts of blood should be avoided as blood renders the examination less satisfactory.

B. Nasal scraping

1. Scrape the mucous membrane covering the cartilaginous portion of the septum with a narrow-bladed scalpel. Spread the material collected on a clean microscope slide. Scrapings should also be made of any lesions in the nostrils, such as nodules or ulcers.

C. Scraped Incision (Wade's Method)

In lepromatus cases, employing a sterilized small double-bellied scalpel blade or new razor blade make a small incision, about 5 mm. long and 2 mm. deep, in suspected nodules and thickened skin. The blade is then turned sidewise and a little tissue scraped from the bottom of the incision and spread on a clean glass slide. Bleeding is avoided if possible, however it is not difficult to see the bacilli in the presence of blood.

PARASITIC EXAMINATION

1. Fresh specimens should be brought into the laboratory in regulation stool containers as soon as possible, and not later than four hours. Ova become contorted and are difficult to recognize unless examined within this period of time.

TUBERCULOSIS

1. A. M. sputum is best after mouth has been rinsed with water.
2. Patients should be instructed to cough. A deep specimen is preferred. Saliva and serous sputa are relatively non-acceptable in making laboratory examinations for B. tuberculosis. Tubercle bacilli are found in the mucous flakes of deep specimens.
3. Only specimens in containers that are supplied by the Tuberculosis Bureau will be examined. Other cans and bottles slow up the work. Sputum containers and mailing containers may be obtained from the Board of Health upon request.
4. No more time than is absolutely necessary should be consumed in the transportation of samples. Old specimens are extremely difficult to handle and also very nauseating.
5. Containers should be neatly labeled, printed in pencil, last name first, given name, source and date.

TYPHOID, PARATYPHOID A & B, BACILLARY DYSENTERY

1. Specimens should be submitted in the stool containers supplied by the laboratory. All labels should follow the procedure as for other specimens.
2. Specimens should be brought into the laboratory within two hours after being placed in the container, unless a preservative has been added. Such preservative is supplied by the laboratory upon request for cases where it will take longer than two hours to submit specimens. The typhoid, paratyphoid and bacillary dysentery groups of organisms are very viable and die quickly after leaving the body.
3. Kracke medium for blood cultures may be obtained in advance for emergency use.

SYPHILIS

A. Blood Examinations for (Wassermann and Kahn Tests)

1. Blood specimens for serologic tests for syphilis are examined daily in the Board of Health laboratories. All specimens submitted to the laboratory before 8:30 a.m. will be included in that day's run.

2. The blood should be taken preferably with a **sterile dry** syringe. (A small quantity of water in a syringe may hemolyze blood. This may be prevented by rinsing the syringe in physiological saline solution or by drying the syringe.)
3. The tube of blood should be slanted and kept at room temperature for one hour to insure proper clotting, and so minimize hemolysis of blood in transit.
4. After the clot has settled (i.e., after blood has been held at room temperature for one to two hours), place blood specimens in ice box until ready for transit to the laboratory. Specimens should not be kept more than four days before they are submitted to the laboratory, and no more time than is absolutely necessary should be consumed in transport. Do not freeze.
5. All specimens must have corks for stoppers (cotton plugs are not permissible). When being sent in the mail, adhesive tape should be wrapped over and around the outside of the cork.
6. Unless Keidel tubes are used, we urge the use of the routine Wassermann tubes which are supplied by the laboratory. The tube should be neatly labeled, printing the last name first, given name, source and date when blood was taken from the patient and purpose for which test is taken (i.e.: prenatal, premarital, pre-employment, treated case, etc.).
7. When mailed, all specimens should be packed securely to prevent breakage in transit and conform with postal regulations.

B. Spinal Fluid

Use chemically clean and sterile cork-stoppered Wassermann tubes as supplied by the Board of Health. Place spinal fluid in cork-stoppered tube. Place ¼ to ⅛ inch wide strip of adhesive tape, about ½ inch long, over top of cork and attach to sides of glass test tube. Tightly wrap another piece of adhesive tape ¼ to ½ inch width, 2½ inches long, about the circumference of the test tube where the cork is inserted, so that the opening between the cork and the test tube is adequately sealed to prevent the loosening of the cork and leakage of the specimen. Spinal fluid thus contained may then be wrapped in a sufficient quan-

tity of surgical cotton, placed in a mailing tube or similar thick-walled container and mailed via parcel post to the laboratory.

URINALYSIS

1. Bring specimens into the laboratory the same day as passed. Urine must be examined while fresh, as decomposition sets in rapidly, especially in warm weather. Preserve by refrigeration until transported.
2. A representative portion of a 24-hour sample, preserved by refrigeration, is preferred. Next in preference is a mixture of the morning and night voidings. Urine taken three or four hours after meals is also satisfactory. Morning voidings are the least desirable.
3. A clean glass container should be used for specimen. A small glass bottle with screw cap is the best for transportation. The laboratory has bottles on hand and will be glad to distribute them in cases where the sample is being brought from a distance.

III. Carriers

DIPHTHERIA

Incubatory Carriers: If found, treat as cases.
Convalescent Carriers: By end of first month 85% of cases are clear of C. diphtheria bacillus. By end of second month 98% are C. diphtheria bacillus free. Virulence test is necessary. Wadsworth found 90% of strains from convalescent carriers are virulent up to three months after recovery.

Contact Carriers: In 10-20% of instances. Virulent in 80% of instances. Average 1 in 1,000 in adults and 2% as true carriers in children.

Possible and Pseudo-Carriers: Discernible by culture and virulence test.

MALARIA

The vegetative forms which produce the symptoms are not infective for the mosquito, but in about 50% of the cases, gametes are found and convalescent or relapsing carriers result. The gametes are more numerous in the chronic than in acute stages of the infection. Therefore, all circumstances are favorable for infection of the mosquito by the carrier. All carriers in malaria belong to the CONVALESCENT group either as convalescent or relapsing carriers. There is a true infection but a balanced parasitism is apparently reached by an antitoxic without an antiparasitical immunity. A special focus exists in the capillaries of the spleen, liver and brain. The balance is delicate and toxic symptoms or frank relapse may occur. Ergot, adrenalin and ultra-violet light have been used to bring the parasites into the peripheral circulation and produce relapses.

MENINGOCOCCUS

Strains from throats of carriers are not as virulent as strains from the spinal fluid of cases. Principal carrier focus is in the vault of the nasopharnyx.

TYPHOID, AMOEBIC DYSENTERY AND BACILLARY DYSENTERY

Temporary Contact Carrier: No demonstrable lesion.
Chronic Contact Carrier: Some definite pre-existing lesion.
Incubatory Carrier: Slight inflammation in tissues; or-

ganisms are found on epithelial surfaces, penetrating between the cells. There is some capillary dilatation and edema and a migration of leukocytes.

Distribution of Typhoid Bacilli in cases:

Greatest percentage in

Blood: - - - -	Middle of 1st week
Urine: - - - -	Middle of 5th week
Feces: - - - -	Middle of 4th week
Duodenal Contents:	Middle of 5th week

Percentage of agglutination reaction is highest in the middle of 5th week.

According to Nichols the distribution percentage of typhoid bacilli in convalescent carriers is as follows:

Feces—4th month 8%	Duodenal contents—4th month 11%
Urine—4th month 1.2%	Agglutination—9th month 11%

In chronic convalescent carriers permanent lesions occur in the ducts of two chief excretory organs—the liver and kidney.

The greatest majority of intestinal carriers are gall bladder or bile duct carriers.

PLACE OF CARRIER IN PREVENTIVE MEDICINE

1. Prevention of spread of parasites from point of multiplication which is usually the host. This procedure involves early treatment in home or hospital, the disinfection of infectious discharges, isolation and quarantine.
2. Prevention of spread of parasites in the environment. This is one of the aims of sanitation and includes disinfection of food, drink, air and the destruction of insects.
3. Personal hygiene for fecal or urinary carriers.
4. Specific immunity by vaccination or serum prophylaxis.

RECOMMENDATIONS FOR:

Respiratory Carriers:

1. Disposal of nose and throat discharges in a safe way, in proper container or handkerchief. Avoidance of promiscuous spitting.
2. Use of personal eating utensils.
3. Washing hands when soiled with respiratory discharges—use of individual towel.
4. Avoidance of close contact in talking and avoidance of kissing.

Genito-urinary Carriers:

1. Avoidance of sexual intercourse unless with protection.
2. Prevention of marriage without permission of physician.
3. Regular treatment.
4. Periodic examinations.

Blood Carriers (Malaria):

1. Avoidance of mosquito bites, by using screens and bed nets.
2. Extermination of mosquitoes found in room (insecticide spray).
3. Following prescribed lines of treatment.
4. Periodic examination.

IV. Amendment to the Postal Laws and Regulations

Diseased Tissues and Other Material of Infectious Nature

ORDER NO. 7831

Section 589 of the Postal Laws and Regulations is amended to read as follows:

589. (a) Specimens of diseased tissues, blood, serum, and cultures of pathogenic microorganisms may be admitted to the mail for transmission to United States, State, municipal or other laboratories in possession of permits referred to in paragraph 2 of this section only when enclosed in mailing cases constructed in accordance with this regulation, provided that bacteriologic or unfixed pathologic specimens of plague and cholera shall not be admitted to the mails except when prepared as hereinafter specifically provided.

(b) Pathologic specimens of plague and cholera which have been immersed for at least 72 hours in four times their volume of 4 percent formaldehyde gas in water, or other fluid of equal or superior disinfecting power for a period sufficient to fix or harden the central portions of the specimen, may be admitted to the mails if packed in the same manner as herein prescribed for other unfixed pathologic tissues (par. 3, subpar. a, b, c).

(c) Cultures and infectious material of plague, cholera, anthrax, undulant fever, and tularemia may be admitted to the mails if enclosed in stout glass tubes sealed by fusion of the glass and packed in a larger stout glass container with a layer of absorbent cotton soaked in 4 percent formaldehyde surrounding the inner container. The outer glass container shall be closed with a rubber stopper or cork of good quality or by fusion of the glass. This double glass container shall then be packed in accordance with the provisions of paragraph 3, subparagraph b and c.

2. No package containing diseased tissue, blood, serum, or cultures of pathogenic microorganisms shall be delivered to any representative of any of the said laboratories until a permit shall have first been issued by the Postmaster General, certifying that said institution has been found to be entitled, in accordance with the requirements of this regulation to receive such specimens.

3. (a) Specimens of sputum, feces, pus, unfixed diseased tissue, or other infectious material fluid in nature or shipped with nondisinfecting fluid shall be placed in stout glass containers of suitable size (but not more than 3 inches in diameter) closed with a metal cover with a rubber, cork, or paraffined paper leak-proof washer or with a cork or rubber stopper of good quality or by fusing the glass, provided that large fixed specimens of diseased tissue may be prepared for shipment outside of mail bags when packed in accordance with the provisions of subparagraph b.

(b) The aforesaid glass container shall then be placed in a cylindrical sheet-metal box, with soldered joints, closed by a metal screw cover with a rubber or felt washer. The vial or test tube in this sheet-

metal box shall be completely and evenly surrounded by absorbent cotton or other suitable absorbent in quantity sufficient to absorb the contents of the glass container should it be broken.

(c) The sheet-metal box with its contents shall then be enclosed in a closely fitting wooden or papier-mache box or tube, at least three-sixteenths of an inch thick in its thinnest part or in a sheet-metal box or tube of sufficient strength to resist rough handling and support the weight of the mails piled in bags. This tube shall be tightly closed with a screw-top cover with sufficient screw threads to require at least one and one-half full turns before it will come off.

(d) Cultures in solid media, blood, serum, spinal fluid, fixed and completely disinfected diseased tissue and infectious materials on swabs shall be transmitted in a stout glass container of suitable size (but not more than 3 inches in diameter) closed with a metal cover with a rubber, cork, or paraffined paper washer or with a stopper of rubber, paraffined cork, or cotton, the last sealed with paraffin or covered with a tightly fitting rubber cap. The tube shall then be packed in a single wooden or papier-mache cylindrical box or tube, at least three-sixteenths of an inch thick in its thinnest part or in a sheet-metal box or tube, of sufficient strength to resist rough handling and support the weight of the mails piled in bags. The glass container in this box or tube shall be completely and evenly surrounded by absorbent cotton or other suitable absorbent packing material. Cultures in media that are fluid at the ordinary temperature (below 45° C. or 113° F.) may be mailed if packed in stout glass vials closed by fusing the glass and enclosed as in subparagraph (b) and (c).

(e) Specimens of blood dried on glass microscopic slides for the diagnosis of malaria or typhoid fever by the Widal test or of other conditions shall be sent in any strong mailing case which is not liable to breakage or loss of the specimen in transit.

(f) Large pathological specimens of fixed diseased tissue and shipments of large numbers of small specimens may be prepared for shipment outside of mail bags. Small specimens of sputum, blood, serum, spinal fluid, pus, feces, fixed or unfixed diseased tissue, or other material fluid in nature or shipped with fluid, forming part of such a shipment shall be placed in stout glass containers as in subparagraph (a) and individually evenly wrapped in absorbent cotton or other suitable absorbent material in sufficient quantity to absorb all the fluid in case of breakage. Large specimens of fixed diseased tissue shall be placed in securely sealed glass containers or in securely closed (hermetically sealed or screw-top or approved patent-top) metal containers with the necessary preservative fluid. The container shall be surrounded by sawdust or other suitable absorbent material to protect against breakage or leakage. Small and large specimens so prepared shall be shipped in a strong securely closed box marked "Fragile—Liquid. This Side Up," or with similar inscription, and be transported outside of mail bags.

4. Upon the outside of every package of diseased tissue, blood, serum, or cultures of pathogenic microorganisms admitted to the mails shall be written or printed the words "Specimen for bacteriological examination. This package shall be pouched with letter mail." Except that large specimens or shipments prepared under paragraph 3, subparagraph (f) shall be marked "Specimen for bacteriologic examination."

W. W. HOWES,
Acting Postmaster General.

V. Current Procedures—Board of Health Laboratories

ACTINOMYCOSIS

METHOD

A. Direct Smear—Spread material over the bottom of a sterile petri dish and look for small white or yellowish ("sulphur") granules about the size of a pin head. Place a granule on a microscope slide by means of a platinum loop, cover with a glass coverslip and press out gently. Examine with high dry or oil immersion. The granule, if actinomycotic colony, will be made of filaments radiating from the center. The terminal ends of the filaments about the periphery will appear club-shaped giving the appearance of light rays radiating from a center (ray fungus).

B. Stained Smear—Prepare thin smear of granule and stain by Gram's method.[33] The central mycellum stains gram-positive and the clubs or bulbous ends are gram-negative.

C. Culture—Incubate 6 to 8 tubes of Sabourad's agar[24] with crushed granules. Incubate from 4 to 5 days at 37° C.—one half aerobically the other half anaerobically (some strains grow only anaerobically). Examine for small, dry, chalky and adherent colonies. Prepare smears and stain by Gram's method.[33] Examine under high dry or oil immersion for gram-positive long filaments with true branching. If no growth is obtained after 5 days, keep culture for two weeks before reporting as negative.

Report as "Positive" or "Negative" for actinomycosis.

ANTHRAX
(B. anthracis)

METHOD

A. Smear—Prepare smears from suspected material, fix, stain by Gram's method[33] and examine for large square ended rods, often encapsulated and with spores (spores are likely to be absent in freshly infected tissue).

Report as: Organisms resembling B. anthracis "Present" or "Absent."

B. **Culture**—Emulsify specimen if necessary. Culture on petri dishes with nutrient agar[20] and flasks of blood broth[5] (50-100 c.c. each). Examine plates macroscopically after 24 hours' incubation at 37° C. for MEDUSA-HEAD colonies.

Report as: Organisms culturally resembling B. anthracis "Present" or "Absent."

C. **Animal Inoculation**—Inoculate guinea pig intradermally and subcutaneously with 0.5 c.c. of emulsion. If the material contains B. anthracis the animal should die in 1 to 4 days with sero-gelatinous edema where inoculated, splenic tumor and darkened heart blood. Smears are made from spleen, liver and heart blood and cultures taken on nutrient agar. Only after evidence of pathogenicity in addition to cultural and microscopic evidence is obtained may the report be given as *"B. anthracis present."*

BOTULISM
(Clostridium botulinus)

METHOD

A. **Culture**—Deep stab a tube of glucose agar ([20] containing 1% glucose), streak plates of nutrient agar[20] and inoculate at least 2 tubes of beef heart broth medium.[3] Incubate anaerobically at room temperature (25° C. is the optimum temperature for Cl. botulinus). On the glucose agar stab growth takes place down the stab with the production of gas which breaks up the medium. On agar plates small translucent colonies appear which are greyish yellow in color and produce delicate off-shoots around the edge. In beef heart broth[3] gas is produced and meat particles which are characteristically reddish usually change to dirty brown or to black. Most cultures have a butyric acid odor.

B. **Animal Inoculation**—Inoculate at least 3 guinea pigs intraperitoneally with 1 c.c. of broth fluid (A) or liquor from suspected food. Use two animals as controls, one receiving 0.5-1.0 c.c. of type A antitoxin and the other 0.5-1.0 c.c. of type B antitoxin. Inoculate a fourth guinea pig with 1 c.c. of the broth fluid or food liquor which has been boiled for 10 minutes (boiling for 10 minutes destroys botulinus toxin). If botulinus toxin is present the animal given untreated liquor together with the one given antitoxin not

specific for the toxin should die within 2 to 96 hours, with a characteristic flabbiness of the abdominal muscles and no gross pathologic changes.

CHANCROID
(Hemophilus ducrey)

METHOD

A. Smear—Pus or gland juice aspirated from buboes before suppuration should be used for smears. Stain by Gram's method[33] and observe for small gram-negative rods, often growing in chains (chains are less likely after ulceration has occurred). The organisms may be intracellular.

Report finding of organisms resembling Ducrey's bacillus.

B. Culture—Employing a warm syringe, gland juice is aspirated from buboes before suppuration and a tube of warm (37° C.) culture medium (1 c.c. sterile rabbit blood, clotted and inactivated by heating at 56° C. for 5 minutes) is inoculated by mixing. Incubate 24 hours in approximately 10% CO_2 at 37° C. Light smokeless candle in air-tight container as for G.C. culturing. Make smears from serum that has collected around clot. Omit heat fixation, but dry smears in incubator (37° C.) for one-half hour. Place smears in distilled water (5 minutes) to remove hemoglobin. Stain by Gram's method. Typical organisms in chains are found. Subculturing to blood agar[4] and incubating 24 hours in 10% CO_2 at 37° C. produces small shiny gray colonies of H. ducrey surrounded by definite zones of hemolysis.

Report cultivation of Ducrey's bacillus.

CHOLERA
(Vibrio cholera)

METHOD

A. Smear—Select flecks of mucous in stool (rice-water stool) and examine by both Gram's stain[33] for gram-negative vibrio forms and by direct examination (wet) for presence of actively motile vibrio forms in mucous particles.

Report as rapidly as laboratory information permits pending confirmation and further laboratory evidence.

B. Culture—Inoculate Dunham's peptone solution (pH 8.4)[10] and incubate 6-12 hours at 37° C. From this

inoculate surface of Dieudonne's medium[9] plates, or nutrient agar[20] plates (pH 8.4) faintly alkaline to phenolphthalein, incubate 18-24 hours. Examine for translucent colonies macroscopically. Stain suspicious colonies and examine microscopically. Check further by specific agglutination, colony appearance, rapid liquefaction of gelatin and cholera red reaction.*

* **Cholera Red Test**—Culture organism in Dunham's peptone solution[10] 2 to 7 days. Add 6 to 8 drops concentrated sulfuric acid. A violet pink color indicates the cholera red reaction.
Note: This reaction is not strictly specific for cholera.

DIPHTHERIA
(Corynebacterium diphtheriae)

METHOD

A. Culture—Culture swabs on fresh Loeffler's medium[16] and incubate 12-24 hours at 37° C. Mix entire growth well with platinum loop and emulsify a very small portion in a drop of distilled water on a microscopic slide. Dry in air, fix, stain with Toluidine Blue[45] (8-10 minutes) and examine for granular, bipolar or barred organisms with clubbed ends.

Report as C. diphtheria "Positive" or "Negative."

B. Smear—After inoculating Loeffler's medium with swab from suspected source, prepare smear by bearing down swab on glass slide to express material for smear. Dry in air, fix, stain with Toluidine Blue[45] (8-10 minutes) and examine for C. diphtheria.

Report as C. diphtheria "Positive" or "Negative."

C. Virulence Tests—To be performed on all positive cultures from convalescents, contacts or suspicious diphtheria carriers. Shave or otherwise remove hair from flanks of two guinea pigs. Inject one with 500 units diphtheria antitoxin. Emulsify the growth from the suspected culture with 5-8 c.c. saline. (If culture is not reasonably pure, streak plate of Tellurite-blood media,[27] incubate 12-24 hours at 37° C., fish at least two colonies thought to resemble C. diphtheria, transfer to Loeffler's[16] slants and incubate 24 hours at 37° C. Examine smear and if purified use emulsion of same.) Inject 0.1 c.c. intracutaneously into each guinea pig. As many as four to eight tests may be done on one animal. The protected animal should show no reaction, but the other animal with a virulent

culture shows redness and induration in 24 hours with superficial necrosis in 48-72 hours.

Report virulence as "Positive" or "Negative."

ENTERIC DISEASES

(Typhoid fever, Paratyphoid fever and Bacillary dysentery) (Cultural Examination)

METHOD

A. **Wet Smear**—Prepare a wet smear preparation made from fresh stool specimen on a glass slide. Examine under low and high dry objectives of microscope. Report presence of pus, blood, mucous shreds and/or macrophages found in specimens of stool submitted for bacillary dysentery.

B. **Culture**—Streak urine or stool specimen on plate of S. S. agar.[26] Incubate 18 to 24 hours at 37° C. Emulsify portion of stool in peptone water—allow suspension to stand 15-30 minutes at room temperature. Streak loopful of urine and/or supernatent fluid from suspension on plate of MacConkey's medium.[17] Incubate 18 to 24 hours at 37° C. Examine plates from A and B for typhoid, paratyphoid and dysentery-type colonies (uncolored and transparent—coli appear as red and opaque colonies, whereas lactose non-fermenting bacteria form colorless colonies). Allow suspicious plates to set at room temperature for 6-8 hours.

C. **Sub-Culture**—Pick suspicious as well as typical colorless colonies. Inoculate Russell's Double Sugar Agar[23] slants, each tube from a single colony by smearing over the surface and stabbing the butt. Incubate 18 to 24 hours (some strains of typhoid may require 30 to 40 hours to produce characteristic reaction). Examine growth on Russell's slants, which show characteristic reaction for enteric disease-inciting organisms, for motility by hanging-drop slide method.

D. **Agglutination:** Complete identification by agglutination test.

METHOD

E. **Macroscopic Slide Agglutination** — Emulsify some growth on a clean glass slide in a loopful of sterile saline. Mix a loopful of this emulsion with a loopful

BACTERIOLOGY REACTION CHART (Enteric Diseases)

BACTERIA	Colony Appearance on S. S. and MacConkeys Agar	Reaction on Russell's Double-Sugar		Motility	Reaction in Media Containing								Indol Production	Reaction in Milk	Serologic Reaction
		Slant	Butt		Dextrose	Maltose	Mannitol	Lactose	Saccharose	Xylose	Rhamnose	Dulcitol			
Eberthella typhosa	uncolored & transparent	N.C.	A	+*	A	A	A	—	—	V	—	A	—	A + / — Alk + / —	Agglutination in E. typhosum anti-serum
Salmonella paratyphi (Paratyphoid A)	uncolored & transparent	N.C.	AG	+	AG	AG	AG	—	—	—	—	—	—	sl. acid to alk	Agglutination in paratyphoid A anti-serum
Salmonella schottmulleri (Paratyphoid B)	uncolored & transparent	N.C.	AG	+	AG	AG	AG						—	sl. acid to alk	Agglutination in paratyphoid B anti-serum
Shigella dysenteriae (Shiga)	uncolored & transparent	N.C.	A	—	A	— or A+ —	—	—	—	—	—	—	—	A+ or — Alk + —	
Shigella ambigua (Schmitz)	uncolored & transparent	N.C.	A	—	A	V	—	—	—	—	A	—	+	A+ or — Alk + —	Agglutination in multivalent (Flexner, Shiga) and/or univalent anti-sera for members of this group
Shigella paradysenteriae (Sonne)	uncolored & transparent	N.C.	A	—	A	A or A+ —	A	A+ —	A+ —	— (A)	A or A+ —	—	—	A or AC	
Shigella paradysenteriae (Flexner)	uncolored & transparent	N.C.	A	—	A	V	A	—	V	—	V	V	V	A+ or — Alk + —	
Shigella sp. (Newcastle type)	uncolored & transparent	N.C.	A or AG	—	A	— or A+ —	— or A+ —	—	—	— or A+ —	—	— or A+ —	—	A+ or — Alk + —	
Shigella paradysenteriae (Hiss)	uncolored & transparent	N.C.	A	—	+	—	+	—	—	V	V	—	—	A+ or — Alk + —	

*—Non motile strains sometimes found, especially when freshly isolated.
N.C.—No change or increase in alkalinity
A—Acid
G—Gas bubbles in medium
V—Variable
Alk.—Alkaline
C—Coagulation

BACTERIOLOGY REACTION CHART (Enteric Diseases)

BACTERIUM	Colony Appearance on S. S. and MacConkeys Agar	Reaction on Russell's Double-Sugar		Motility	Reaction in Media Containing								Indol Production	Reaction in Milk	Serologic Reaction
		Slant	Butt		Dextrose	Maltose	Mannitol	Lactose	Saccharose	Xylose	Rhamnose	Dulcitol			
Shigella alkalescens	uncolored & transparent	N.C.	A	–	A	A	A	–	–	A	A	A	+	A to Alk	
Shigella paradysenteriae (Strong)	uncolored & transparent	N.C.	A	–	+	–	+	–	–	V	V	+	+	A+ or – / Alk + –	Agglutination in B. dysenteriae Strong anti-serum. Not agglutinated by anti Shiga, Hiss or Flexner
Bact. dispar	uncolored & transparent	N.C.	A	–	A	A	A	A+ –	V	V	V	V	+	A or AC	of questionable value
Salmonella enteritidis	uncolored & transparent	N.C.	AG	+*	AG	AG	AG	–	–	AG (–)	AG	AG (–)	–	A to Alk	Agglutination in multivalent and/or univalent anti-sera for members of this group
Salmonella suipestifer	uncolored & transparent	N.C.	AG	+*	AG	AG	AG	–	–	AG (–)	AG	AG (–)	–	A to Alk	
Salmonella typhimurium (aertrycke)	uncolored & transparent	N.C.	AG	+*	AG	AG	AG	–	–	AG (–)	AG	AG (–)	–	A to Alk	
Alkaligenes fecalis	uncolored & transparent	N.C.	N.C.	+	No Sugars								–	Alk	of questionable value
Proteus morganii	uncolored & transparent	N.C.	A or AG	+*	AG	–	–	–	V	–	–	–	+	– or Alk	of questionable value
Escherichia coli	red and opaque	A	AG	V	AG	V	AG	AG	V	V	V	V	AV	A or AC	of questionable value
Aerobacter aerogenes	uncolored & transparent	A	AG	+	AG	AG	AG	AG	AG				–	A or AC	of questionable value
Staphylococcus sp. (albus)	uncolored sl. opaque or transparent	N.C.	A	–	A	A	A	A	A	V	V	V	–	AC	of questionable value

*—Non motile strains sometimes found, especially when freshly isolated.
N.C.—No change or increase in alkalinity
A—Acid
G—Gas bubbles in medium
V—Variable
Alk.—Alkaline
C—Coagulation

of diluted agglutination serum (dilution determined to be specific by standardization) on glass slide. The drop of emulsion serves as a control, remaining homogenous in appearance. Tilt slide for 2 to 3 minutes by hand. Definite agglutination will usually occur with species analogous to that against which the serum was produced, while the bacilli of other species will seldom be agglutinated. Read agglutination slides as —, 1, 2, 3, or 4 plus depending upon the number of organisms clumped rather than on the size of the clump. The results of this test require confirmation.

F. **Macroscopic Tube Agglutination**—Employ typhoid, multivalent dysentery, or multivalent Salmonella agglutinating sera, depending upon the reaction in the Russell's double-sugar slant, the motility and the result of the slide agglutination test. Use sera of known specificity, the titre having been previously determined. Use 0.5 c.c. of anti-serum dilutions in each tube and one control tube of 0.5 c.c. saline (0.85%) without anti-serum.

Place 0.9 c.c. saline (0.85%) in the first test tube of a series and 0.5 c.c. in each successive tube. Add 0.1 c.c. of agglutinating serum to the first tube (Dilution 1:10), mix well by drawing fluid up and down pipette. Transfer 0.5 c.c. from first tube to second tube and mix by drawing up and down pipette (dilution 1:20). Continue the diluting process from the second to the third tube and up to and including the next to the last tube. Discard the 0.5 c.c. from the last dilution. The last tube serves as the control.

Emulsify the growth of an 18-24 hour Russell's slant culture in 1 to 3 c.c. of normal saline by rotating the tube or by rubbing of the growth with a sterilized platinum loop. Place a small pledget of sterile cotton (from the lower end of a sterile cotton plug) into the top of the tube and push to the bottom with a sterile pipette. Draw the suspension through the cotton and place into a sterile tube. **Use cotton-plugged pipettes for all transfers.** Dilute with normal saline to approximately the density of the typhoid antigen used in the agglutination tests (so that newsprint letters can just be seen through the test tube and its suspension contents).

Add 0.5 c.c. of the culture to each tube of the serum dilutions (total volume 1 c.c.) and **flame the upper**

1/3 of the tube thoroughly. This makes a total dilution of 1:20 in the first tube, 1:40 in the second tube, 1:80 in the third tube, etc. 1:20, 40, 80, 160, 320, 640, 1280, 2560, 5120). Incubate overnight at 37° C. Read. Freshly isolated strains of dysentery bacilli, especially Sonne, Schmitz and Newcastle types may be agglutinated only partially or not at all in multivalent dysentery serum.

Submit reports on all specimens as promptly as possible. If the final report cannot be made within a week, make a preliminary report.

Report as "Negative," "Positive" **(name of organism)** and highest dilution giving agglutination. i.e.: Positive S. flexner 1:5120; or the result of the examination was considered unsatisfactory. Any other significant information concerning the examinations should also be reported.

SEROLOGIC CLASSIFICATION OF ENTERIC DISEASES

Antiserum	Bacteria Agglutinated	Bacteria NOT Agglutinated
Normal serum (Human)	Salmonella schottmulleri (1:40-1:80) Shigella flexner (1:40-1:80) Salmonella paratyphi (1:10 to 1:20) Shigella hiss (1:40-1:80)	Eberthella typhosa Salmonella paratyphi Shigella enteritidis Shigella shiga
(Animal)	Shigella hiss (1:40-1:80)	Shigella shiga
Eberthella typhosa	Eberthella typhosa Shigella flexner (1:40-1:80)	Eberthella tarda Salmonella annamensis Salmonella schottmulleri (paratyphoid B)
Eberthella oedematiens	Eberthella oedematiens	Eberthella typhosa Salmonella annamensis
Salmonella aertrycke	Salmonella aertrycke Salmonella schottmulleri (paratyphoid B) Salmonella morganii	Salmonella enteritidis Salmonella schottmulleri (paratyphoid B) Eberthella typhosa

SEROLOGIC CLASSIFICATION OF ENTERIC DISEASES

Antiserum	Bacteria Agglutinated	Bacteria NOT Agglutinated
Salmonella enteritidis (Gaertner's bacillus)	Salmonella enteritidis	Salmonella schottmulleri (paratyphoid B) Salmonella aertrycke Eberthella typhosa Salmonella annamensis Salmonella hirschfeldii
Salmonella morganii	Salmonella morganii (of questionable value)	
Salmonella paratyphi (paratyphoid A)	Salmonella paratyphi (human infection 1:20-1:40) Eberthella typhosa (1:20-1:40)	Salmonella annamensis Salmonella schottmulleri (paratyphoid B)
Salmonella schottmulleri (paratyphoid B)	Salmonella schott-mulleri (1:20-1:240) Salmonella aertrycke	Eberthella typhosa Salmonella paratyphi Salmonella enteritidis
Salmonella annamensis	Salmonella annamensis	organisms of genus Eberthella organisms of genus Shigella organisms of genus Escherichia
Shigella flexner	Shigella flexner Shigella hiss Eberthella typhosa (1:40-1:80)	Shigella shiga Shigella strong Shigella schmitz
Shigella hiss	Shigella hiss Shigella flexner	Shigella shiga Shigella strong
Shigella schmitz	Shigella schmitz	Shigella shiga Shigella flexner
Shigella shiga	Shigella shiga Shigella flexner Shigella hiss } Coagglu-tinins	Shigella (strong, alkalescens, dispar, schmitz, and hiss)
Shigella strong	Shigella strong	

SEROLOGIC CLASSIFICATION OF ENTERIC DISEASES

Bacterium	Agglutinated By	Are NOT Agglutinated By
Shigella alkalescens	Alkalescens anti-serum	Shiga anti-serum
Shigella dispar	Dispar anti-serum	Shiga anti-serum
Shigella flexner	Normal human serum (1:40-1:80) Flexner anti-serum Shiga anti-serum Human serum from case of Flexner (minimum 1:160) Human serum from cases of Shiga (Shigella dysenteriae) Typhoid anti-serum (1:40-1:80)	Schmitz anti-serum
Shigella hiss	Normal human serum (1:40) Hiss anti-serum Shiga anti-serum Human serum from case of Hiss (1:80 or above) Human serum from case of Flexner	Shiga anti-serum
Shigella schmitz	Schmitz anti-serum	Shiga anti-serum Flexner anti-serum
Shigella shiga	Human serum from human case (1:80 to 1:160 maximum)	Normal human serum Normal animal serum Hiss anti-serum Human serum from case of Flexner Schmitz anti-serum
Shigella strong	Strong anti-serum	Shiga anti-serum Flexner anti-serum Hiss anti-serum

AGGLUTINATION TESTS ON PATIENTS' SERA

METHOD

A. Preparation of Antigens—

1. Prepare "H" antigens for detecting "H" agglutinins against E. typhosa, Paratyphosa A (S. paratyphi), Paratyphosa B (S. schottmulleri), Shigella antigens (Flexner V, W, X, Y, & Z), and shigella dysenteriae (Shiga). Use formalized suspensions of a smooth motile strain except for Shiga (although this is a non-motile organism it is prepared the same as an "H" antigen). Inoculate surface of sterile veal infusion agar in blake bottle with 0.5-0.7 c.c. of a 24-hour broth culture of the organism from which the antigen is to be made. Incubate Blake bottle in inverted position 18 to 24 hours at 37° C. Wash off growth with 5 c.c. of formalized (0.5 per cent) physiological saline (1.25 c.c. formaldehyde per 10 c.c. of sterile saline). Store suspension 7-10 days in refrigerator to kill bacteria in suspension. Test for sterility by transferring one loopful of formalized bacterial suspension to a tube of infusion broth—incubate 48 hours and check for sterility. This suspension may be considered as a stock concentrate and stored in the refrigerator until needed. To dilute stock antigen for use add sterile saline (0.85 per cent) until a suspension comparable to the McFarland nephelometer #3 is obtained (printing just is visible when looking through a tube of suspension of the proper density). Check suspension against analogous antisera of known titer.
2. Prepare "O" antigens for detecting "O" agglutinins against E. typhosa, Proteus OX2 and Proteus OX19 in the same manner as above, but using a non-motile strain of the organism.

B. Macroscopic Agglutination Test—Set up a series of 10 clear small test tubes (Wassermann or agglutination tubes). Add 0.9 c.c. of saline to the first tube and 0.5 c.c. to the remaining seven tubes. Add 0.1 c.c. of the patient's serum to the first tube and mix well with a 1 c.c. graduated serological pipette. Transfer

0.5 c.c. of this serum-saline mixture to the second tube and mix well. Transfer 0.5 c.c. of the serum-saline mixture in the second tube to the third tube and mix well. Continue until 0.5 c.c. of the serum-saline mixture has been removed from the ninth tube and discard this last 0.5 c.c., leaving the tenth tube as a control. To each of the ten tubes add 0.5 c.c. of the antigen suspension. The final serum dilutions with saline and antigen suspension are 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120 and control. Shake the rack containing the tubes and incubate at 37° C. for 20 to 24 hours.

Read agglutination as 4, 3, 2, and 1 plus as indicated by decreasing amounts of agglutination and increasing cloudiness of the supernatant fluid. "H" agglutinins produce large flakes of the floccular type which are easily broken. "O" agglutinins produce granular or small-flaking agglutination.

AGGLUTINATION TESTS
(Interpretation and Reports)*

The interpretation of agglutination titers with somatic "O" or flagellar "H" antigens has the same limitations as those obtained with living antigens. The significance of certain titers depends on such factors as: (1) duration of patient's illness, (2) history of previous inoculations with typhoid-paratyphoid vaccine, (3) variability in the production of agglutinins in different individuals and (4) non-specific stimulation of typhoid-paratyphoid agglutinins by other organisms (anamnestic reaction). Information on the first two of these factors should be furnished as part of the case history while the effect of the latter two factors can only be borne in mind for consideration under certain circumstances.

If the patient has not been recently vaccinated (within six months), and "O" titer of 1:160 or greater is indicative of typhoid fever. In early typhoid cases, the "O" titer generally appears first and increases faster than the "H" titer. With reactions due to antityphoid inoculations, the "H" titer is usually much higher than the "O" ("O" rarely above 1:80) and persists longer.

Only the highest dilution giving a 2+, or greater, agglutination reaction shall be reported.* Report simply as + (not 2+, 3+ or 4+) and give dilutions. Dilutions giving

* Complete records must be kept on all dilutions made, whether positive or negative.

doubtful reactions (1+ or +—) should not be reported. See illustration below.

Agglutination Reactions

	1:40	1:80	1:160	1:320	1:640
Typhoid "O"	4+	4+	2+	+	—
Paratyphoid B "H"	+	—	—	—	—
Undulant Fever	+	—	—	—	—
Tularemia	—	—	—	—	—

Report as follows:

Agglutination reaction: Typhoid 1:160. No reaction for paratyphoid B, undulant fever or tularemia.

Comment: Reaction serologically positive for typhoid fever. Stool specimens should be taken for confirmation of diagnosis and later specimens should be submitted to determine possible carrier state.

Where the reactions for typhoid and other diseases are not clear-cut and it is impossible to know which ones are significant, give reactions and request another specimen. For example,

Agglutination Reactions

	1:40	1:80	1:160
Typhoid "O"	2+	—	—
Paratyphoid B "H"	1+	—	—
Undulant Fever	2+	—	—
Tularemia	—	—	—

Would be reported as:

Agglutination Reactions: Typhoid 1:40, undulant fever 1:40. No reaction for tularemia or paratyphoid B.

Comment: Reactions too weak to be diagnostic. Suggest another blood specimen be submitted for possible rise in titer.

Chylous and hemolyzed specimens

In reporting negative reactions on dilutions which are chylous	No comment necessary.
In reporting positive reactions on dilutions which are chylous	Reaction is probably unreliable since specimen is chylous. Request another specimen taken at least 3 hours after eating.
Hemolyzed specimens run at 37° C.	Specimen hemolyzed and result may be unreliable. Request another specimen be submitted.

Typhoid

(a) PATIENT ILL, NOT RECENTLY (six months and longer) GIVEN ANTITYPHOID INOCULATIONS

"O" titer 1:160 or greater	Positive serological reaction for typhoid. Suggest that stool specimen be taken for confirmation of diagnosis and later specimens submitted to determine possible carrier state.
"O" titer 1:40 or 1:80	Reaction too weak to be diagnostic, another specimen should be submitted to determine possible rise in titer.

(b) PATIENT ILL, RECENTLY (within six months) GIVEN ANTITYPHOID INOCULATIONS

"O" titer 1:160 or greater	Reactions may be due to antityphoid inoculations. Further specimens of blood should be submitted to determine possible rise in titer. A specimen of stool should also be sent in a proper container for confirmation of diagnosis.
"O" titer 1:40 or 1:80	Reaction too weak to be diagnostic. Reaction may be due to antityphoid inoculations. Request that another specimen be submitted to determine possible rise in titer.

(c) WELL PERSON, FOR CARRIER

"O" titer 1:40 or higher	Reaction may or may not indicate carrier state. If person has been inoculated against typhoid within the past six months, reaction may be due to inoculation. Specimens of stool and urine should be submitted.
(d) Specimens submitted for test of immunity following antityphoid inoculation	Agglutination titers are of little, if any, value as a test for immunity to typhoid. There is no simple test by which immunity can be measured.

Paratyphoid A "H"

Agglutinins form slowly in paratyphoid A infection. A reaction of 1:20 for paratyphoid A in the absence of reactions for typhoid and paratyphoid B is "slightly suggestive." A reaction of 1:40 or higher is "suggestive." Other specimens should be submitted to determine if there is any rise in titer. Stool specimens should always be examined from all cases of suspected paratyphoid fever.

Paratyphoid B "H"

(a) PATIENT ILL, NOT RECENTLY GIVEN ANTITYPHOID INOCULATIONS

"H" titer 1:160 or higher (reactions lower for typhoid)	Reactions suggestive of paratyphoid B. Another blood specimen should be submitted to determine possible rise in titer. A specimen of stool should also be submitted in a proper container.
"H" titer 1:40 or 1:80 (reaction negative for typhoid)	Reaction too weak to be diagnostic. Another specimen of blood should be submitted to determine possible rise in titer.

Dysentery

Titer 1:40 or higher. Titer should be the highest obtained with any of the Flexner antigens.	Reaction only suggestive of dysentery. Unless a marked rise in titer is obtained with successive specimens, a reaction of even 1:320 or 1:640 may be without significance.
V 1:80; W 1:40; X 1:40; Y 1:160; Z —	The final diagnosis should depend upon isolation of the organism from the stool.

Report: Flexner 1:160
Sonne 1:40

Negative Reports

If onset is of recent date: Agglutination reactions may not be positive until a week or ten days after onset. If history is suggestive, another specimen should be submitted.

If history is not filled out: Agglutination reactions may not be positive until a week or ten days after onset. If history is suggestive another specimen should be submitted.

Reference:—"Current Standard Procedures"—Maryland State Department of Health.

EPIDEMIC OR STREPTOCOCCUS (Septic) SORE THROAT

METHOD:

A. **Culture**—If swab is submitted, streak surface of two blood agar[4] (rabbit) plates, surface of a Loeffler's[16] slant and inoculate a tube of semi-solid agar.[25] Incubate one blood agar[4] plate under 5 to 10% carbon dioxide and the other under normal atmospheric conditions for forty-eight hours at 37° C., unless hemolytic colonies appear earlier. Incubate the Loeffler's[16] slant and tube of semi-solid agar[25] 18 to 24 hours at 37° C., make smears of mixed culture of each, stain with methylene blue[38] or toluidin blue[45] and examine with microscope under oil immersion.
(If morphologically typical diphtheria bacilli are found, report in usual manner.)

CLASSIFICATION OF STREPTOCOCCI

ALPHA	BETA	GAMMA
Small, raised, convex and opaque. Surrounded by a narrow, usually less than 1 mm. zone of hemolysis and may or may not show green discoloration around the colonies.	Small, white, hard, opaque and surrounded by well-defined zone of hemolysis. The colonies may be pushed intact over the surface of the agar and break up when touched with the inoculating needle.	Produce no hemolysis or discoloration of red blood cells. Rarely encountered in routine diagnostic bacteriology. Most strains which appear gamma in 24 hours will produce alpha hemolysis if the plates are re-incubated for 24 hours and then refrigerated overnight.

B. **Smear**—Make smear of swab after inoculating culture media. Stain by Gram's method.[33] Also make smears of streptococcus-like colonies and stain by Gram's method.[33] Alpha streptococci vary in size, are definitely elongated, are often pleomorphic and may show bacillary swollen forms. Chains may be observed from solid as well as liquid media and within a single chain the individual cocci may vary in size and shape. Beta streptococci occur in pairs, groups and very short chains and are usually small, round with their adjacent sides slightly flattened and morphology quite regular. Long chains are seen in smears from broth cultures, but never from solid media.
Report as Hemolytic Streptococci "Present" or "Absent." Any other significant information concerning the examinations should also be reported.

FILARIASIS

METHOD:

A. **Wet Method**—The filaria parasites are most numerous in the peripheral circulation at night. Best time for examination is between 10 p.m. and 2 a.m. Ascepticize finger or ear lobe, puncture with sterile stylus and place large drop of blood on slide. Immediately cover with cover glass and examine with microscope under the low power lens. The microfilaria are slender, about as wide as erythrocytes, 0.2 to 0.4 millimeters long and can be located by the disturbance they produce among the corpuscles.
Report microfilaria "Present" or "Absent."
Should above examination fail to reveal filaria larvae, proceed with following method.

B. **Concentration Method**—Collect 1 c.c. of blood in 5 c.c. of 2 per cent acetic acid. Mix well and centrifuge. Discard supernatant. Spread sediment on slide, cover with cover glass and examine with low power lens.
Report microfilaria "Present" or "Absent."

C. **Staining Method**—Make thick blood smear (stir to defibrinate blood) or a smear from the sediment obtained by the concentration method (B). Dry in air, fix and stain by Wright's[48] or Giemsa's[32] stain.
Report microfilaria "Present" or "Absent."

D. **Chylous Urine**—Centrifuge specimen of urine, make smear of sediment and stain by Wright's[48] or Giemsa's[32] stain. Examine under oil immersion.
Report microfilaria "Present" or "Absent."

FOOD POISONING

METHOD: (Also see Botulism)

A. **Food**—Consider the evidence with regard to typhoid, dysentery or chemical intoxication. Otherwise, examine for salmonella and staphylococci. Make smear and stain by Gram's method.[33] Streak plates of MacConkey[17] and blood agar,[4] incubate at 37° C. 18 to 24 hours. Also inoculate portion of food in tubes of veal infusion broth,[2] incubate at 37° C. 12 to 24 hours and streak MacConkey[17] and blood agar[4] plates from this. If significant numbers of staphylococci are noted, isolate on blood agar[4] to determine pigment and hemolysis.

Non-lactose fermenting organisms on MacConkey agar[17] should be fished and inoculated into Russell's[23] and identified by fermentation reactions and specific agglutinations as for typhoid, paratyphoid and bacillary dysentery.

(See Enteric Diseases)

B. **Stools**—Handle as for typhoid, paratyphoid and bacillary dysentery—isolation and identification of organism. *(See Enteric Diseases)*

C. **Blood**—(1) Culture as for typhoid, paratyphoid and bacillary dysentery.

(2) Agglutination tests on serum as for typhoid, paratyphoid and bacillary dysentery. *(See Enteric Diseases)*

Report: Organism (type or characterization) found.

Note: Food poisoning may not always be of bacterial origin, nor can all be traced by laboratory methods. Poisonous animal foods (mussels); vegetable foods (toadstools, solanin (sprouted potatoes), ergot from grain, etc.); chemical poisoning (preservatives, arsenic, lead, tin, copper, etc.) and bacterial toxins (botulinus, staphylococcus, etc.) must be considered as well as food allergies, forms of indigestion, intestinal parasites, intestinal symptoms in typhoid fever, the dysenteries and other enteric diseases.

Submit all suspected **foods** and specimens of **vomitus, feces** and **urine** to the Board of Health as soon as possible.

FUNGUS INFECTIONS

Mycoses of Skin, Mucous Membranes and Internal Organs

METHOD:

A. **Smear**—On a microscope slide emulsify an ample portion of pus or scrapings in 20% sodium or potassium

hydroxide. Cover with a glass and allow to stand 20 to 30 minutes. Examine cleared specimen under microscope.

B. **Culture**—Pus specimens or swabs may be inoculated directly on Sabouraud's agar.[24] Scrapings may be treated with 70% alcohol for a short time to somewhat reduce the contaminants. Incubate at room temperature several weeks.

MONILIA (THRUSH) AND BLASTOMYCETES (Blastomycosis)

Smear—Monilia are usually seen as oval bodies, but mycelia and conidia may also be found. Blastomycetes are a closely allied group, which are seen as round or oval bodies. (M. albicans—THRUSH; M. Psilosis—SPRUE)

Culture—On Sabouraud's agar[24] blastomycete produces a thick, rather wrinkled growth.

SPOROTRICHA

Smear—Closely resembles yeast, but forms long thin branching septate hyphae.

Culture—On Sabouraud's agar[24] sporotricha produce a growth which is first gray and feathery, but later becomes raised, wrinkled and dark brown in color.

RINGWORM OR TINEA

Microspora (small spored type)—Common case of ringworm of the scalp. Variety most frequently affecting children is known as Microsporon audouini. Long branching hyphae along with large numbers of irregularly arranged small spores, with which the infected hairs become covered at end in the sheaths. Grows on nutrient agar as a thick mass with hyphae spreading into the air. May be white, brown or orange in color.

Trichophyta (large spored type)—More common in infections of nails, hair and in ringworm of the beard. The "spores" are larger and probably not true spores at all, but short vegetative cells. In the infected parts the so-called spores are arranged in rows and are thus differentiated from microsporon. On nutrient agar[20]

or Sabouraud's agar[24] some species produce feathery colonies and others harder wrinkled growths. Most are white, but some varieties may produce pigment —violet, red, etc.

FAVUS
(Achorion schonleinii)

Smear—The scutula are formed by masses of the fungus with irregularly arranged spores in the central parts and hyphae of irregular appearance toward the periphery.

Culture—Grows very slowly on Sabouraud's,[24] taking weeks (2 to 4) to develop. Growth varies in appearance. Some colonies sometimes have a curiously twisted appearance of the surface while others may show a fluffy appearance from the shooting out of aerial hyphae.

GONORRHEA
(Neisseria gonorrhea)

METHOD:

A. **Stained Smear**—Stain fixed smears by Gram's method.[33] Examine under low and high dry for suspicious areas (microscopic fields showing white cells). Then examine with oil immersion for gram-negative intracellular diplococci resembling N. gonorrhea.

Report as: (1) Gram-negative intracellular diplococci **"Present"**; **"Suspicious"** or **"Indeterminate-repeat"** with culture, and as **"Negative."**

(2) Leukocytes (WBC) as **"occasional," "few," "moderate," and "many."**

(Occasional=5 or less, few=5 to 30, moderate=30 to 50, many=50 or more per field).

(3) Abundant bacterial flora.

B. **Culture**—Streak the material on coagulated-blood (chocolate) agar[8] plates. Place in air tight container in inverted position to prevent flooding by the water of syneresis (erroneously called water of condensation), place a lighted smokeless candle in the container. Put the cover on the container and seal with adhesive or Scotch drafting tape. Incubate at 35° C. for about 48 hours. Examine plates for small grayish opaque colonies with undulated margins (N. gonorrhea). When no gonococcus colonies are observed by direct inspection, the "oxydase" reaction is applied

by tilting the plate and flooding half the plate with 1 or 2 c.c. of a 1% aqueous solution of p. aminodimethylaniline monohydrochloride. The plate is observed every 1 or 2 minutes for a period of 15 minutes for evidence of change in the color of the colonies. The series of color reactions, pink, maroon and black, readily identifies the colonies of Neisseria. The time required for the color changes varies from 6 to 10 minutes. Smears from pink colonies showing gram-negative diplococci and sub-cultures on carbohydrate media serve to complete the identification of the gonococcus. Since the dye component is toxic for the gonococcus, sub-cultures should be made as soon as a suspected colony becomes pink. When oxidation has progressed until the colony is black, the cells are usually dead and sub-cultures fail to grow. The dye does not interfere with subsequent Gram stains.

Cultural identification on the gonococcus is completed by testing their fermentation reactions on 1% dextrose, lactose, maltose and saccharose (in either ascitic agar[1] slants with Andrade's indicator* or Difco Phenol-red broth).

* 0.5% acid fuchsin in distilled water decolorized to a yellow tint by adding Normal NaOH. One c.c. of sterile indicator solution is employed for each 100 c.c. of media.

Differentiation of Neisseria

	Dextrose	Maltose	Succrose	Lactose
N. gonorrhea	+	−	−	−
N. intracellularis	+	+	−	−
N. catarrhalis	−	−	−	−
N. sicca	+	+	+	−

Report cultures as "Positive," "Indeterminate-repeat," or "Negative."
Note: If the specimen is over 3 hours old when received and "Negative" results are obtained, report as "Unsatisfactory."

INFECTIOUS MONONUCLEOSIS

METHOD (Heterophile antibody test):

Inactivate serum 15 minutes at 56° C. Set up a series of eight Wassermann or serological tubes as for agglutination test. In an additional test tube mix 2.1 c.c. saline and 0.3 c.c. inactivated serum (1:8 dilution). Place 1 c.c. of the 1:8 dilution in the first tube and 1 c.c. of saline (0.85 per cent) in each of the remaining test tubes. Add 1 c.c. of the 1:8 dilution to the second tube and mix well. Using a graduated 1 c.c. pipette transfer 1 c.c. of this serum-

saline mixture in the third tube. Mix well and transfer 1 c.c. of this serum-saline mixture to the fourth tube. Continue in this manner through the seventh tube discarding 1 c.c. of this serum-saline mixture, keeping the eighth tube as a control.

Add 1 c.c. of a 2% sheep cell suspension to each tube. Use sheep cells for the suspension that are at least 24 hours old and not older than one week. (Final dilutions are 1:16, 1:32, 1:64, 1:128, 1:256, 1:512 and 1:1024.) Mix well by shaking for 2 to 3 minutes. Centrifuge 2 minutes at high speed. Read agglutination by tapping test tube with finger. Complete agglutination or clumping of the sheep cells is read as a four plus. Decreasing amounts of agglutination with increasing turbidity are read as three, two and one plus reactions.

Report highest dilution, giving definite agglutination (2 plus or above) 1:64 dilution or higher may be considered a positive serological reaction for infectious mononucleosis. Report lower titers (1:16 and 1:32) as doubtful but suggestive serological reaction for infectious mononucleosis and request another specimen. Any increase in titer of over two dilutions during the course of the disease may be considered significant.

Note: Should an apparent infectious mononucleosis case with a history of serum treatment give a high titer, conduct an adsorption test with guinea pig kidney, as follows: At 37° C. for one hour with frequent stirrings adsorb 1 c.c. of a 1:5 dilution of the serum with 0.25 c.c. of a finely ground and washed emulsion of guinea pig kidney. Centrifuge mixture and test serial dilutions for presence of sheep cell agglutinins. If the original sheep cell titer of the serum is not significantly reduced by the adsorption, heterophile agglutinins of infectious mononucleosis are present. If sheep agglutinins are adsorbed with guinea pig kidney, heterophile antibodies of the infectious mononucleosis type are absent.

INTESTINAL PARASITES
(Protozoa and Helminths)

AMOEBIC DYSENTERY (Endamoeba histolytica)

METHOD:

A. Direct Smear—1. With a small brush or applicator make a thin film of emulsified fresh (preferably warm) stool in physiological saline on glass microscope slide. Cover with coverslip and examine microscopically for amoebic cysts using low and high dry objectives. Reports are made on stained preparations made from stools showing cysts in fresh smears. Or—2. A fleck of stool is mixed with a drop of saline and streaked across a glass slide. A cover glass is then placed over one side of the smear. On the other half of the slide a drop of iodine-eosin stain is mixed with the streaked material. A second cover glass is

placed over the stained part of the smear and both parts are then examined microscopically. On the unstained side, the organisms are found in their natural coloring, while on the stained side, the internal structures are clearly brought out.

B. Stained Smear—On clean flamed microscope slides prepare a smear of the stool specimen. In series of coplin jars treat slide as follows:

1. Immerse wet smear in Schaudinn's fixative[43] overnight or hold at 60° C. for 10 minutes.
2. 70% alcohol (70 c.c. 95% alcohol made up to 95 c.c. with distilled water) tinged to wine color with Gram's Iodine solution[33] for 10 minutes.
3. 70% alcohol for 5 minutes.
4. 50% alcohol (50 c.c. 95% alcohol made up to 95 c.c. with distilled water) for 5 minutes.
5. 35% alcohol (35 c.c. 95% alcohol made up to 95 c.c. with distilled water) for 5 minutes.
6. Running tap water for 2 minutes.
7. Rinse with distilled water for few seconds.
8. Iron Hematoxylin[37] for 5 to 10 minutes.
9. Running tap water for 10 minutes.
10. Rinse with distilled water for few seconds.
11. Differentiate in 2% aqueous iron-alum[36] (control degree of differentiation with the microscope).
12. Running tap water for 20 minutes.
13. 35% alcohol for 5 minutes.
14. 50% alcohol for 5 minutes.
15. 70% alcohol for 5 minutes.
16. 95% alcohol for 5 minutes.
17. 100% (absolute) alcohol for 5 minutes.
18. Carbol-Xylol[29] for 5 minutes.
19. Xylol for 5 minutes.

Examine for forms of E. histolytica under high dry and oil immersion; or mount with a thin layer of balsam[39] for permanent slide and examine under high dry or oil immersion. For purposes of control and record when E. histolytica is found prepare permanent mounts.

Report as **"Positive"** or **"Negative"** for E. histolytica. Report any other significant information concerning the examination. If other protozoa are found report such as **"E. coli," "chilomastix," "giardia," "trichomonas,"** etc.

COMPARISON OF ENDAMOEBA HISTOLYTICA WITH TWO COMMON NON-PATHOGENIC AMOEBAE

Vegetative Forms

	Endamoeba histolytica	Endamoeba coli	Endolimax nana
Size	Variable; 20-40 microns.	Constant; 20-30 microns.	Very small; about 8 microns.
Motion	Active and directional.	Sluggish.	Active but soon lost.
Structure	Sharp differentiation between ectoplasm and endoplasm.	Poor differentiation between ectoplasm and endoplasm.	Poor differentiation between ectoplasm and endoplasm.
Contents	Red blood cells.	Bacteria but no blood cells.	Bacteria but no blood cells.
Nucleus	Indistinct or not seen.	Easily seen.	Indistinct.

Cystic Forms

Size	Variable; 7-15 microns.	Large.	Small; 7-9 microns.
Structure	Spheric or ovoid. Single occasionally double cyst wall.	Usually double cyst wall.	Ovoid, very thin walls.
Cytoplasm	Granular chromidia in younger forms	Large masses of glycogen (brown with iodine). Chromidia rare.	Little glycogen. No chromidia.
Nuclei	4 in mature cysts.	8-16; rarely 4.	4; very small.

HELMINTHS

METHOD:

A. **Direct Smear**—Place drop of physiological saline on microscopic slide, emulsify portion of stool by use of wooden applicator, cover with glass coverslip. Examine under low and high dry powers. Report actual parasite identified.

B. **Concentration of Ova**—1. DeRivas technique—Emulsify 1 gram of feces in 5 c.c. of 5% acetic acid by shaking in a stoppered test tube (use broken glass or beads if necessary). Permit to stand ½ to 1 minute and filter through doubled cheese-cloth into another test tube. Mix filtrate with equal volumes of ether by shaking. Centrifuge few minutes. The mixture will separate into 4 distinct layers. The ether extract at the top is removed and used for the occult blood test. Below the ether is a detritus plug (bile, soaps and protein). Underneath this is the acetic solution and at the bottom of the tube there is a small amount of sediment. Collect the sediment in a capillary pipette, place it on a microscope slide, cover with a cover glass and examine with microscope (low or high dry power lens).

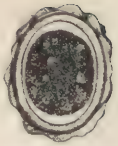
Report ova of parasite identified.

2. Flotation method—In a small sputum jar or similar container (approximately ¾" in diameter and 2" high) emulsify a portion of stool (approximately 2 grams) in a super-saturated solution of sodium chloride. Carefully add the sodium chloride solution till the meniscus of the stool-saline suspension reaches the top level of the jar. Place a microscope slide over the top of the jar so that a very small air bubble is formed between the surface of the solution and the underside of the glass slide (the level of the saline-stool suspension may be controlled with a glass dropper). Allow to stand 20 to 30 minutes, remove the slide from the jar in a rapid upward motion, cover with a glass coverslip and examine under the microscope for ova of parasites.

Report as "Positive" and give name of ova identified, or as "Negative."

C. **Occult Blood Test** (Benzidine)—Freshly prepare a small amount of benzidine in glacial acetic acid (saturated solution). Mix with equal portion hydrogen

EGGS OF PARASITES FOUND IN THE HUMAN



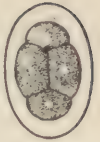
Ascaris lumbricoides
(Fertilized)
ROUND WORM

Ascaris lumbricoides
(Unfertilized)
ROUND WORM

Trichocephalus trichiuris
WHIPWORM

Enterobius vermicularis
(Oxyuriosis)
HUMAN PINWORM
OR SEATWORM

Necator Americanus
(Uncinariasis)
AMERICAN HOOKWORM



Ancylostomia duodenale
"OLD WORLD" HOOKWORM

Taenia saginata
BEEF TAPEWORM

Taenia solium
PORK TAPEWORM

Dipylidium caninum
DOG TAPEWORM

Diphyllobothrium latum
BROOD (FISH) TAPEWORM

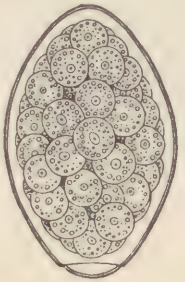
Hymenolepis nana
(Taenia murina)
DWARF TAPEWORM

Hymenolepis diminuta
RAT TAPEWORM

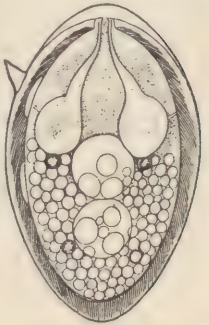
Clonorchis sinensis
CHINESE LIVER FLUKE

Schistosoma haematobium
VESICAL BLOOD FLUKE

Schistosoma japonicum
ORIENTAL BLOOD FLUKE



Fasciolopsis buski
LARGE INTESTINAL FLUKE



Schistosoma mansoni
MANSON'S BLOOD FLUKE

peroxide (5% H_2O_2—U.S.P.). A few c.c. of this mixture are added to an equal quantity of the ether extract (Part B). A blue color appears in the presence of hemoglobin.

Report as "Positive" or "Negative" for occult blood.

D. **Anal Swabs** (For Enterobius ova)—A small square of cellophane is wrapped about the tip of a glass rod and held in place by a rather wide rubber band. The swab is used in a firm stroking motion, directed outward from the anal opening, parallel to and entering the folds of skin of the entire perianal region. The best time for swabbing is early morning before defecation and bathing. Eggs of helminths and sometimes the entire worm are picked up by the swab. For examination the rubber band is cut, the cellophane with the adherent material is spread on a glass slide in a few drops of physiological saline, an additional drop of saline is placed on top of the cellophane, covered with another slide, pressed together and examined under the microscope with the low power objective. At least seven swabs obtained on different days should be examined before a negative diagnosis is warranted.

LEPROSY
(Mycobacterium leprae)

METHOD:

A. **Stained Smear**—Prepare smear on slide from nasal scrapings, lesion or biopsy tissue. Stain by steaming with carbol-fuchsin[49] for 5 minutes or by leaving slide immersed in carbol-fuchsin at room temperature overnight. Rinse in running water, decolorize with 3% nitric acid or 25% muriatic acid until the color ceases to flow (few seconds). Watch decolorization of smear against a white background. Rinse in running water. Counterstain with methylene blue[38] 10 to 30 seconds. Rinse in running tap water, drain dry and examine under oil immersion for Hansen's bacillus (B. leprae). The organisms resist acid decolorization and resist acid alcohol must less than do tubercle bacilli. B. leprae are often shorter and straighter than M. tuberculosis and are often found in clusters in "lepra cells."

Report in terms of acid-fast organisms resembling B. leprae "Present" or "Absent." The presence of Hansen's bacilli in "lepra cells" may be reported, if warranted.

METHOD:

Stained Thick Smear—Touch the undersurface of a meticulously clean, unscratched and non-corroded microscope slide to the crest of a large drop of blood and, without losing contact with the drop of blood, move the slide in narrow circles until a smear about the size of a dime is made. The ideal thick film is several layers of erythrocytes thick in the middle and has a thinner edge of one-cell thickness. Lay the slide flat and allow to dry in air. Protect from dust and insects.

Stain film preparation (thick smear) for malarial parasites by Giemsa's[32] method.

Report results of the examination as: Malarial parasites "Present" or "Absent."
Report any other significant information concerning the examination.

1. **Benign Tertian Parasite (Plasmodium Vivax)**—The asexual cycle, which takes 48 hours, can be observed in the peripheral blood. The infected cell becomes enlarged, loses its characteristic staining reaction and shows stippling with pink-staining granules (Schuffner's granules). The merozoites produced by schizogony average about twenty and are grouped together irregularly. The early sexual forms (gametocytes) are round in shape.

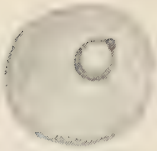
2. **Quartan Parasite (Plasmodium Malariae)**—The asexual cycle, which takes 72 hours, can be observed in the peripheral blood. The infected cell does not enlarge and Schuffner's granules are absent. The merozoites average about eight and are arranged like a rosette. The gametocytes are round, but smaller than those of the benign tertian parasite.

3. **Malignant Tertian Parasite (Estivo Autumnal Parasite, Plasmodium Falciparum)**—The asexual cycle probably requires 24 to 48 hours but occurs in the internal organs and cannot be followed in the peripheral blood. The infected cell is not enlarged and shows red staining granules (Maurer's granules). The number of merozoites produced varies considerably and may be as many as thirty. The gametocytes are quite different from those of the other two malarial parasites, being crescent shaped. As with the other two parasites they are found in the peripheral blood. When these are seen the diagnosis of the type is certain. Differentiation may be difficult in the early tro-

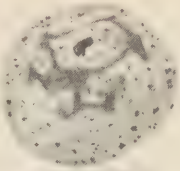
MALARIA PARASITES

BENIGN TERTIAN

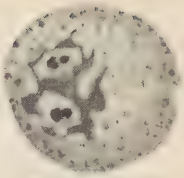
NORMAL RED CELL
(note size relationship)



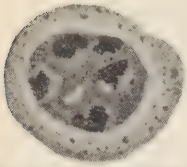
TROPHOZOITE
(ring form)



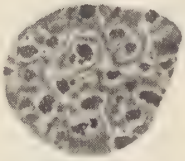
TROPHOZOITE
(older form—
Schuffner's dots present)



YOUNG SCHIZONT
(nuclei dividing)

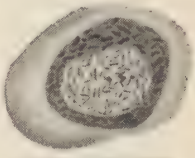


SCHIZONT (more advanced)
(brownish pigment present)



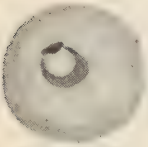
MEROCYTES
(segments poorly defined)

MACROGAMETOCYTE
(female)
(chromatin compact)

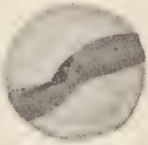


MICROGAMETOCYTE
(male)
(chromatin diffuse)

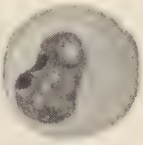
QUARTAN



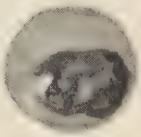
TROPHOZOITE
(ring form)



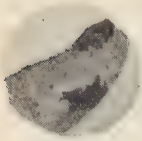
TROPHOZOITE
(characteristic band form)



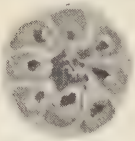
TROPHOZOITE
(oval form—
nucleus dividing)



SCHIZONT
(young, binuclear form)

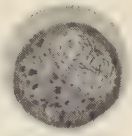


SCHIZONT
(band form)



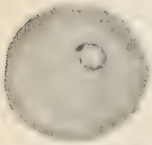
MEROCYTES
(characteristic daisy form)

MACROGAMETOCYTE
(female)
(chromatin compact)



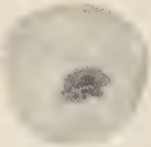
MICROGAMETOCYTE
(chromatin diffuse)

MALIGNANT TERTIAN (Estivo-Autumnal)

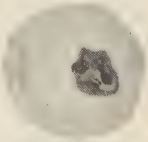


TROPHOZOITE
(single ring form)

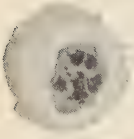
TROPHOZOITES
(multiple infection)



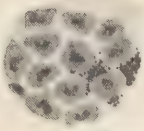
TROPHOZOITE
(older form)



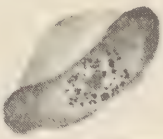
SCHIZONT
(young binuclear form)
rarely found in circulating blood



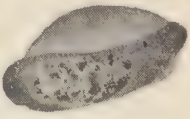
SCHIZONT
(older form)
rarely found in
circulating blood



MEROCYTES
rarely found in
circulating blood



MACROGAMETOCYTE
(female)
(characteristic crescent shape)
(chromatin compact)



MICROGAMETOCYTE
(male)
(crescent shape—
chromatin diffuse)

phozoite stage, but multiple infection of normal sized red cells by small ring forms is very suggestive of the malignant tertian variety.

MENINGOCOCCUS MENINGITIS
(Neisseria intracellularis)

METHOD:

A. Cerebrospinal Fluid Cell Count—Perform immediately after spinal tap, if to be of value. Place fluid directly, without dilution, in counting chamber. Count total number of cells in 9 large squares, multiply result by 10/9 to obtain number of cells in 1 c.c. If cells are too numerous by this method, dilute fluid in "white count" pipette as for a regular leukocyte count.

Report number of white cells per cubic millimeter (cu. mm.).

B. Smears—As soon as possible prepare smear as for differential blood count. With cloudy fluid make direct smear. For clear fluids centrifuge, discard supernatant fluid and make two smears of the centrifuged sediment.

1—**Differential count:** Stain one smear with Wright's stain[48] (as for blood smear). The predominance of polymorphonuclear leukocytes will aid ruling out tubercular meningitis, poliomyelitis, encephalitis and lymphocytic chorio-meningitis.

2—**Gram stain:** Stain the remaining smear by Gram's method,[33] examine under oil immersion for gram-negative diplococci within and without the leukocytes.

C. Culture—Using centrifuged sediment or uncentrifuged spinal fluid when meningococci seem to be especially abundant, inoculate the surface of a blood agar plate,[4] ascitic agar[1] slant and a tube of semi-solid agar[25] (depending on nature of spinal fluid, use 0.5 to 1 c.c. of inoculum). (Do not overlook spinal fluid as a medium.) Incubate remaining fluid by itself. Incubate cultures and spinal fluid overnight at 37° C. and examine. Meningococci appear on ascitic agar[1] and on blood agar[4] as smooth and translucent colonies with or without confluent growth. On semi-solid agar[25] they grow as a pellicle at the surface. The only other member of the genus Neisseria that

produces translucent colonies is the gonococcus (N. gonorrhea). These are usually smaller, growth is less luxuriant and there is less tendency to confluence. Phenol red sugar broths inoculated with organisms from isolated meningococci colonies, produce acid in dextrose and maltose. The N. gonorrhea produces acid from dextrose only and N. catarrhalis ferments none at all.

D. Serologic Reaction—The identity of the meningococcus should be confirmed by agglutination. Growth not more than 24 hours old from a solid medium is used. Make macroscopic slide agglutination tests from typical colonies. Emulsify part of a colony on a glass slide in a drop of saline (0.85 per cent). On same slide place a loopful of multivalent anti-meningococcus typing serum diluted 1:10 and one loopful of saline. Add to each of these one loopful of the suspension. Mix by rotating slide 3 to 5 minutes. If organisms are meningococci, definite clumping will appear in anti-meningococcus serum but not in saline control. Stain film preparation by Gram's method[33] to determine whether agglutinated organisms are gram-negative cocci. Confirm results with macroscopic tube agglutination test.

Cultures for this purpose may be obtained by inoculating blood agar[4] or ascitic agar[1] slants from some of the colonies and incubating them for from 16 to 18 hours at 37° C. Use varying dilutions from multivalent anti-meningococcus serum (from 1:100 through 1:2000 will usually prove adequate for diagnostic purposes). Incubate at 55° C. for 24 hours. Determine serological group by further agglutination tests.

Report N. meningococcus and type present, or N. meningococcus "Absent."

If gram-negative cocci that do not agglutinate are present, report what has been found and send a transplant of the culture to the Board of Health, Bacteriological Laboratory, Honolulu, for confirmatory tests.

PERTUSSIS
(Hemophilus pertussis)

METHOD:

A. Culture—Prepare plates of Bordet-Gengou agar[6] for cough plates (inoculated by exposing open plate a few inches from mouth of patient during characteris-

tic deep cough). Incubate at 37° C. for 48 to 72 hours. H. pertussis appears as opaque, grayish (mercury-like) colonies. Differentiate from Pfeiffer's bacillus by growing on media containing blood (H. pertussis will grow on blood media, Pfeiffer's bacillus will not).

B. **Rapid Agglutination Test**—In capillary tube containing citrated-methylene blue and pertussis antigen, (Lilly) draw up ¼ inch of patient's blood (from finger or ear lobe) and expel on porcelain or glazed paper card. Mix blood and antigen mixture with applicator or toothpick. Mix by tilting in rotary motion for one minute. Observe for agglutination. Negative reactions show no clumping. Positive reactions appear with a small amount of (2 plus) to pronounced (4 plus) clumping.

Report as "Negative" or "Positive."

PLAGUE
(Pasteurella pestis)

METHOD:

A. **Stained Smears** — Prepare smears on microscopic slides from spleen, blood, nodes, bone marrow of infected animals, fluid aspirated from human buboes, or from sputum (pneumonic type). Dry in air, fix by heat and stain 10 to 30 seconds with Wayson's stain.[47] (Organisms may survive the staining process and care is mandatory.) Plague bacilli appear as short stubby rods with bipolar granules. Confirm by animal inoculation.

Report as organisms resembling B. pestis "Present" or "Absent."

B. **Culture—CAUTION: Notify local health officer and await permission prior to culturing B. pestis.** Inoculate surface of agar[4, 20] in petri dish and incubate at room temperature (20 to 28° C.) for 48 hours. B. pestis appears as slimy, small, flat, grayish, delicate colonies. Also inoculate surface of MacConkey's medium[17] (to eliminate Salmonella infections of rodents simulating plague). If septicemia is suspected, culture 5 to 10 c.c. of blood in a flask of veal infusion broth[2] or Kracke's medium.[13] Fish suspicious colonies; make smears from same. Fix in flame and stain by Gram's method[33] or, fix in flame and stain with Wayson's stain[47] for 10 seconds. Wash in run-

ning tap water. Allow to drain and dry in air. Examine with oil immersion. **BE CAREFUL NOT TO HANDLE STAINED SMEAR WITH HANDS AS ORGANISMS MAY BE VIABLE.** Cover smear with Canada Balsam[39] and cover glass, if slides are to be shipped between laboratories.

Employing portions of the suspicious colonies, inoculate tubes of the following media:

Dunham's peptone[10]
Dunham's peptone[10] solution with Brom thymol blue indicator* containing 1% glucose.
Dunham's peptone[10] solution with Brom thymol blue indicator* containing 1% galactose.
Dunham's peptone[10] solution with Brom thymol blue indicator* containing 1% lactose.
Dunham's peptone[10] solution with Brom thymol blue indicator* containing 1% glycerin.
Dunham's peptone[10] solution with Brom thymol blue indicator* containing 1% Rhamnose.
Litmus milk[15]

* 1 c.c. of a 1.6% alcoholic solution per liter.

B. pestis is non-motile and produces acid only with glucose and galactose.

Pasteurella	Motility after 18 hrs. in broth at 28° C.	Glucose	Galactose	Lactose	Glycerin	** Indol	Litmus Milk Reaction
P. Pestis	—	A	A or NC	NC	NC	—	NC or slight A
P. Pseudo-tuberculosis	+	A	A	A or NC	A	—	Alk.
P. Aviseptica	—	A	+	NC	NC	+	NC

A—Acid
NC—No change
Alk.—Alkaline

**Indol test—Inoculate tube of Dunham's peptone[10] (5 c.c.) and incubate 18 to 24 hours. Add 0.2 to 0.3 c.c. of reagent (dissolve 5 grams of para dimethylaminobenzaldehyde in 75 c.c. amyl alcohol (reagent) and add 25 c.c. concentrated hydrochloric acid). Shake and let it stand for about a minute. Dark red color in amyl alcohol layer is "Positive" for Indol.

C. **Animal Inoculation**—Inoculate two guinea pigs, one percutaneously, by rubbing suspected material gently on the shaven, slightly scratched abdominal surface, and the other subcutaneously. Place inoculated animals in individual glass jars rimmed with vaseline on the upper part of jars' inner surface and cover with fine mesh gauze. Infection in the guinea pig

is first noted by loss of appetite and sensitivity to external stimuli (snapping of finger or tapping outside of jar). Death usually occurs in 3 to 8 days. If guinea pig does not die in 10 days, sacrifice. Autopsy guinea pig and examine for enlarged reginal lymph nodes (buboes), subcutaneous edema and hemorrhage at site of inoculation and small necrotic foci in the spleen (mottled spleen), liver, lungs and other organs. Prepare smears from suspected organs or tissue and stain with Wayson's stain[47] (as in Part A).

Report organisms resembling B. pestis present or absent. The identity of B. pestis must be proved culturally.

PNEUMONIA
(Diplococcus pneumoniae)

METHOD:

A. **Stained Smear**—Gram stain[33] a smear made from a bit of thick sputum. If "positive," microscopic observation (oil immersion) will reveal the presence of encapsulated lance-shaped diplococci.

B. **Pneumonia Typing (Neufeld or Quellung Test)** — Preferably using a specimen of sputum showing six to ten pneumococci per field, place six flecks of sputum on portions of glass slides. Mix each thoroughly with two or three volumes of the respective pneumococcus typing sera (Polyvalent, groups A to F). Mix one loopful of methylene blue[38] with each mixture of sputum and serum. Cover with a glass slip and examine under oil immersion for swelling of capsule (positive Quellung). Having determined which group serum reacts specifically, repeat the test with type specific (monovalent) pneumococcus typing sera.

Report as "Positive" or "Negative." If "Positive" report the type found.

C. **Mouse Inoculation**—If organisms are too scarce for direct typing inject a mouse with 0.5 to 1.0 c.c. intraperitoneally. Four to six hours later remove a droplet of peritoneal fluid from the mouse and stain by Gram's method[33] to determine if mouse is to be sacrificed (abundance of pneumococci). When sufficient organisms are present, perform the Neufeld test (B) with the peritoneal fluid from the mouse.

Report as "Positive" or "Negative." If "Positive" report the type found.

POLIOMYELITIS
(Infantile paralysis)

METHOD:

A. Cell Count (Spinal Fluid)—Employing a fresh specimen, accurately make a spinal fluid cell count.
Report actual cell count in numbers of cells per cubic millimeter (cu. mm.) of spinal fluid.

B. Qualitative Sugar (Benedict's)—see under Urinalysis.

C. Globulin (Cerebrospinal fluid)—see under Syphilis.

D. Colloidal Mastic (Cerebrospinal fluid) — see under Syphilis.

"Q" FEVER

METHOD:

A. Macroscopic Agglutination Test—Use "Q" fever antigen undiluted or diluted 1:2 and conduct test as for routine blood agglutination test.

B. Complement-Fixation—Dilute antigen as per titer (present lot may be diluted 1:16), and conduct Kolmer technique complement-fixation test on inactivated blood serum.

Report as "Positive" or "Negative" for "Q" fever.

Note. This test is conducted at the Central Laboratory only (Board of Health, Honolulu).

RAT-BITE FEVER
(Spirillum minus)

METHOD:

A. Animal Inoculation—Bacteriological diagnosis is best made by inoculating guinea pigs, mice or rats subcutaneously or intraperitoneally with blood taken at the height of a febrile paroxysm or with serum expressed from a local lesion. In the human patient the organism is present in the swollen local lesion, the focal lymph glands or the blood, but is difficult to demonstrate except by animal inoculation. S. minus is found in the kidney at autopsy.

B. Darkfield—Examine aspirated peritoneal fluid or blood from inoculated animals daily from the 5th to 15th day by darkfield illumination. (Employ darkfield examination technic described under "Syphilis.")

C. Stained Smear—Prepare smears for Spirillum minus. Allow to dry spontaneously (the smear may be fixed by heating in flame, but is not essential). Stain 2 minutes with Perrin's stain[42] while heating (be care-

ful that stain does not dry out). Stain 6 minutes at room temperature, wash by immersing slide in container of water and by moving it gently. Dry in air. Examine under high dry and oil immersion. *Report as S. minus "Present" or "Absent."*

SEPTICEMIA
(Whole Blood Culture for Bacteremia)

METHOD: **Blood Culture Procedure**

A. Make a blood-agar pour plate by removing 5 c.c. of sample as received in the Kracke medium[13] and adding to it 10 c.c. infusion agar (45° to 50° C.) to which 1 c.c. sheep or rabbit blood has been added. Incubate plate 5 days. Record readings.

B. Add 3 to 5 c.c. of sample as received in Kracke medium[13] to a tube of Brewer's medium. Incubate until 11th day.

C. Incubate original bottle of Kracke medium[13] (45-50 c.c.) to which blood (5 c.c.) had been added until 11th day, if negative.

Note: Examine plates and cultures daily macroscopically for turbidity and other evidences of growth and microscopically by Gram stained[33] smears. For typhoid and other enteric pathogens subculture on selective media (ie: MacConkey[17] or S.S.[26]).

If "Positive," send report with identity of organism immediately. If "Negative," send preliminary report after 48 to 72 hours' incubation, and final report after 10 days' incubation. Whenever obvious contaminants are isolated, request another specimen.

* Brewer's medium—Sodium thioglycollate medium with dextrose and Eh indicator (Baltimore Biological Laboratory).

SYPHILIS
(Treponema pallidum)

METHOD:

A. **Darkfield**—Place chancre fluid on clean microscope slide and cover with vaseline rimmed cover slip (to keep preparation from drying). Examine with microscope equipped for darkfield (darkfield sub-stage and funnel placed inside of oil immersion objective). The following technique is recommended as a standard procedure: Lower the sub-stage and place a drop of immersion oil, free of bubbles on the upper surface of the condenser. Put the slide on the stage and center the specimen. Raise the sub-stage until the oil

is spread by contact with the slide, filling the space between the slide and container. Examine for Treponema pallidum under low and high dry. By employing the low and high dry objectives, time is saved in locating the organism and in most instances provides adequate results for diagnostic purposes. As a further check on an organism under view, the field may be centered, a drop of immersion oil[34] placed on the coverslip and examination made with the oil immersion objective.

Report as Treponema pallidum "Present," or "Absent" and any other significant information concerning the examination.

B. Serology—Directors wishing to have their laboratories approved for serologic tests for syphilis may, upon proper application to the Board of Health, obtain information relative to the requirements and procedures for serologic evaluation.

Serologic tests are performed in strict compliance with the techniques of their individual authors, as outlined in U. S. Public Health Service Supplement #9, 1938, and Supplement #11, 1940 to Venereal Disease Information.

Report results of blood tests as being either "Positive," "Doubtful," "Anticomplementary," or "Negative."

Report quantitative blood tests by numbers representing the dilution of the serum with saline as compared to the amount of serum in the qualitative test as unity.

Example:

Kolmer Quantitative Test on Blood Serum

Tube No.	Actual Amount of serum in tube	Dilution factor	Report as comparative portions of reagin
Qualitative	0.2 c.c.	1	1
Quantitative			
#1	0.1 c.c.	½	2
#2	0.05 c.c.	¼	4
#3	0.025 c.c.	⅛	8
#4	0.005 c.c.	1/40	40
#5	0.0025 c.c.	1/80	80

Report last tube giving 3 or 4 plus reaction in terms of whole number.

Example:

Serum Tube	Reading	
0.1 c.c.	4 plus	
0.05 c.c.	4 plus	Report as Quantitative
0.025 c.c.	4 plus	Kolmer—Positive 8
0.005 c.c.	Neg.	
0.0025 c.c.	Neg.	

If last tube gives a 2 reaction interpolate or take median number

Example:

Serum Tube	Reading	
0.1 c.c.	4 plus	
0.05 c.c.	4 plus	Report as Quantitative
0.025 c.c.	4 plus	Kolmer—Positive 25 (strongest
0.005 c.c.	2 plus	reaction is between 8 and 40)
0.0025 c.c.	Neg.	

C. Spinal Fluid:

Report quantitative spinal fluid tests by amount of spinal fluid in each dilution and the amount of reaction in each tube.

Example:

c.c. of spinal fluid	Reaction
0.5 c.c.	4 plus
0.25 c.c.	4 plus
0.125 c.c.	3 plus
0.0625 c.c.	2 plus
0.03125 c.c.	Neg.

D. Mastic Test for Spinal Fluids (Colloidal Mastic Test for Cerebrospinal Fluids (Cutting)

1. **Mastic Solution**—Dissolve 10 grams of gum mastic N. F. VI in 100 c.c. absolute alcohol and filter. To 2 c.c. of this stock solution add 18 c.c. of absolute alcohol, mix well and pour rapidly into 80 c.c. of distilled water.
2. **Alkaline Saline Solution**—Prepare a 1.25 per cent solution of sodium chloride in distilled water and to each 99 c.c. of the solution add 1 c.c. of 0.5% of potassium carbonate (C.P. anhydrous) in distilled water.

3. **Test**—Arrange six small test tubes in rack. Place 1.5 c.c. alkaline saline in first tube and 1 c.c. in each of remaining five tubes. Add 0.5 c.c. spinal fluid to the first tube, mix thoroughly and transfer 1 c.c. to the second tube. Mix thoroughly and transfer 1 c.c. from the second tube to the third and so on until the fifth tube, from which 1 c.c. is discarded. The sixth tube remains as a control. To each tube add 1 c.c. of mastic solution. Mix well, incubate at 37° C. for 6 to 12 hours or overnight (12 to 24 hours) at room temperature.

Report tube reactions in series, reporting positive reactions as "Trace," "3," "4," and "5," as indicated by the formation of precipitate in the tubes. (i.e., 5, 5, 3, tr, 0 or 00000).

E. Globulin (Qualitative Detection of Increased Amounts of Protein in Cerebrospinal Fluid)—In a small test tube add approximately 1 c.c. carbolic acid, liquid (90%) (liquified phenol U.S.P.) or 1 c.c. of a 10% aqueous solution of phenol (Pandy's technique). Gently overlay surface of liquified phenol with 0.3 to 0.5 c.c. of spinal fluid. If "positive," a bluish white ring or cloud is formed at point of contact between phenol and spinal fluid.

Report as Globulin—"Positive," "Trace," or "Negative."

TETANUS
(Clostridium tetani)

METHOD:

A. Stained Smear—Make film preparation of suspected material (pus or tissue scrapings from the suspected wound, emulsified in a small amount of physiological saline). Stain by Gram's method and examine for characteristic "drum-stick" spores of Cl. tetani.

Report as spores resembling those of Cl. tetani "Present" or "Absent."

B. Culture—Inoculate specimens in veal infusion medium[2] (pH 7.4-7.8) and on blood agar,[4] and nutrient agar[20] plates (pH 7.4-7.8). Incubate anaerobically at 37° C. for 72 hours. Examine for typical bacilli. If spores are present in the fluid medium, heat the culture to between 75° and 80° C. for 30 minutes to kill the non-sporing organisms and inoculate blood agar[4] plates for the isolation of pure colonies. Study

organisms for cultural and biochemical characteristics. (No acid or gas in carbohydrate media. Indol positive. Nitrates not reduced. Litmus milk[15] shows no change or slow precipitation of casein.)

C. **Animal Inoculation**—Mix a portion of the original material (A) or of heated culture (B) with sterile emery dust and inject subcutaneously into the thigh of a 250 gram guinea pig. Results are usually more satisfactory if a broth suspension of a pure culture is used. A control guinea pig protected by an intraperitoneal inoculation of tetanus antitoxin should receive a similar injection at the same time. If Cl. tetani is present the unprotected animal will develop tetanus and die within one to four days.

TRICHINOSIS
(Trichinella spiralis)

METHOD:

A. **Examination of Blood**—Larvae are sometimes found between the 6th and 22nd day after onset of symptoms. Obtain 10 c.c. blood (venous puncture) and mix thoroughly with 25 c.c. of 2 per cent acetic acid. (Larvae are also sometimes found in the spinal fluid after centrifuging). Centrifuge at high speed 15 to 30 minutes, pour off supernatant, examine sediment, either moist by placing a drop on slide covered with cover slip, or by Wright stained[48] smears.

B. **Examination of Muscle**—Tease out bits of tissue removed from the diaphragm, pectoralis major, deltoid, biceps or gastrocnemius muscles in a drop of physiological saline, or compress small piece of muscle firmly between two glass slides. Cover with cover glass and examine with high dry or oil immersion for trichinella cysts. The coiled larvae are easily seen.

C. **Enzymatic Digestion**—Place about ½ pound of lean pork, taken, if possible, from the diaphragm or masseter muscle, in 300 to 500 c.c. of digestion mixture.[41] Stir constantly in 37° C. bath 3 to 5 hours. Skim off any fat that has formed. Filter mixture through fine mesh sieve (20-gauge). Filter slowly 1 to 2 minutes or centrifuge. Allow filtrate to sediment. Examine sediment under microscope using low power objective. Living forms coil and uncoil.

Report "Presence" or "Absence" of larvae of Trichenella spiralis.

TRICHOMONIASIS
(Trichomonas vaginalis)

METHOD:

A. **Wet Preparation**—A hanging drop preparation of the suspected secretion from the vagina is examined with microscope employing the low and high dry objectives. Occasionally, if the organisms are few or their motility is suspended, a drop of 0.1 per cent safranine can be added to the pus as a diluent. The nuclear material and protoplasm are stained, but the trichimonads remain unstained and conspicuous against a pale pink background. The stain appears to stimulate the motility of the trichomonads.

B. **Darkfield**—Darkfield examination of Trichomonas vaginalis will show the flagella and undulating membrane.

TUBERCULOSIS
(Mycobacterium tuberculosis)

METHOD:

A. **Smears**—Make smear from representative portion of specimen. For sputum, if possible, use "cheesy lumps" of lung exudate. In cerebrospinal fluid or other body fluid make smear from coagulum or fibrin web. When coagulum or fibrin web is not present and in the examination of urine, centrifuge the specimen at high speed for 30 minutes and prepare smear from the sediment. Dry in air and fix in flame. Stain the preparation by Ziehl-Neelsen's method.[49]

Report acid-fast bacilli present or absent (indicate number of Gaffky scale).

Gaffky	I	only 1 to 4 bacilli in whole preparation
"	II	1 bacillus on average in many fields
"	III	1 bacillus on average in each field
"	IV	2 to 3 bacilli on average in each field
"	V	4 to 6 bacilli on average in each field
"	VI	7 to 12 bacilli on average in each field
"	VII	13 to 25 bacilli on average in each field
"	VIII	about 50 bacilli on average in each field
"	IX	about 100 bacilli on average in each field
"	X	innumerable bacilli on average in each field

B. **Culture**—(Sputum, urine, gastric content, etc.)—Concentrate and digest specimen in a centrifuge tube by mixing the specimen with an equal part of a 5 per cent aqueous solution of oxalic acid. Incubate for 30

minutes at 37° C. Add 2 to 3 drops of brom-thymol blue (0.04 per cent) as an indicator. Titrate to neutral (green color) with Normal sodium hydroxide solution. Centrifuge at high speed for 30 minutes. Discard supernatant and employ sediment for culture, smear and/or animal inoculation.

With platinum loop smear portion of sediment over slant of Petragnani's medium.[22] Incubate at 37° C. in horizontal position for 24 hours to allow inoculum to adhere to medium. Continue to incubate at 37° C. in vertical position. Examine each culture macroscopically at 7 to 10 day intervals. At this time remove the screw cap or paraffined stopper for an instant and replace it to allow air to enter. Hold all negative cultures three months before discarding. If typical colonies of acid-fast bacilli are found between the 10th to 25th day after or later, follow with guinea pig inoculation.

A preliminary report should be forwarded to the physician—"Acid-fast bacilli have been found in culture. A guinea pig is being inoculated to determine if these are tubercle bacilli. A report will be made later."

C. **Animal Inoculation**—Inoculate guinea pigs with sediment of concentrate or with saline emulsion of suspicious acid-fast culture. Inject guinea pig subcutaneously in the inguinal region. Examine guinea pigs when cultures are "positive" or six weeks after inoculation. Autopsy animal as indicated by loss of weight or palpable glands. Smears are made from any lesion and stained for acid-fast bacteria. Examine inguinal glands, axillary glands, retroperitoneal glands, spleen, liver, mediastinal glands and lungs. Hold animal for three months when loss of weight is not noticeable and glands are not palpable—then autopsy.

Report as follows: Tubercle bacilli "Present" or "Absent." Any other significant information concerning the examinations should also be reported.

TULAREMIA
(Bacterium tularensis)

METHOD:

A. **Slide Agglutination Test**—On large glass slide ruled in squares or on glass slides with depressions (hang-

ing drop slides) using a 0.2 c.c. pipette graduated in .001 c.c. place serum as follows:

1. 0.08 c.c. serum
2. 0.04 c.c. serum
3. 0.02 c.c. serum
4. 0.01 c.c. serum
5. 0.005 c.c. serum
6. 0.0025 c.c. serum
7. no serum (control)

Add to each amount of the serum 0.03 c.c. of standard formalin-treated suspension of Bacterium tularense giving the following dilutions:

1. 1:20
2. 1:40
3. 1:80
4. 1:160
5. 1:320
6. 1:640
7. control

Mix sera and antigen with toothpick or wooden applicator, starting with dilution 1:640 dilution and working to the 1:20 dilution. Shake slide slowly in rotary movement for 2 to 3 minutes. Read agglutination over artificial light above black background.

B. **Tube Agglutination Test**—Prepare dilutions of serum in saline (0.85 per cent) from 1:10 to 1:1280. Combine equal quantities of the dilutions (0.5 c.c.) and killed suspension of Bacterium tularense. If using Sharpe & Dohme concentrated suspension dilute concentrate 1:50 (0.1 c.c. antigen plus 4.9 c.c. saline). Final dilution 1:20 to 1:2560. Incubate the test tubes four hours at 56° C. or overnight at room temperature. Read for macroscopic agglutination. Titers over 1:160 are suggestive (previous or latent infections may leave titers). Report highest dilution in which definite agglutination occurs (2 plus).

Interpretation

Titer 1:160 or greater	Positive serological reaction for tularemia.
Titer 1:40 or 1:80	Reaction too weak to be diagnostic. Another specimen should be submitted to determine possible rise in titer.

When there is cross agglutination with the Brucella group a tularemia titer of 1:160 is suggestive. Otherwise a titer of 1:80 is suspicious.

Note:
Save serum and also test for B. abortus or B. pestis if negative for tularemia. Titers must be carefully determined since there is a degree of cross agglutination with Brucella organisms.

TYPHUS FEVER
(Rickettsia prowazeki var. mooseri)

METHOD:

A. **Agglutination Test**—(Weil-Felix)—Performed with Proteus 0X19. See under Enteric disease agglutination test.

Report highest agglutination titer of patient's serum with Proteus 0X19. Example: 0X19 1:1280.

Interpretation of Agglutination Test for Typhus Fever and Spotted Fever

Titer 1:160 or greater	Positive reaction for typhus or spotted fever.
Titer 1:40 or 1:80	Reaction too weak to be diagnostic. Another specimen of blood should be submitted to determine possible rise in titer.

Note:
Typhus (Brill's Disease) is endemic in Hawaii, whereas, there is no available history as to the occurrence of spotted fever. **Proteus** 0X19 should be used as the antigen for typhus or Proteus 0X2 as the antigen for spotted fever. A reaction with either of these antigens in the titers indicated above should be reported.

UNDULANT FEVER
(Brucella abortus)

METHOD:

A. **Rapid Test (Huddleson)**—On large glass slide ruled in squares or on glass slides with depressions (hanging-drop slides) using a 0.2 c.c. pipette graduated in .001 c.c. Place serum as follows:

1. 0.08 c.c. serum
2. 0.04 c.c. serum
3. 0.02 c.c. serum
4. 0.01 c.c. serum
5. 0.005 c.c. serum
6. 0.0025 c.c. serum
7. No serum (control)

Add to each amount of the serum 0.03 c.c. Huddleson's antigen giving the following dilutions:

1.	1:20	These dilutions are arrived at on the basis of the amount of serum in 2 c.c. as used in a test tube. The water (2 c.c.) is omitted, but the amounts of serum, antigen and electrolyte are the same and the reactions correspond to those in the test tubes.
2.	1:40	
3.	1:80	
4.	1:160	
5.	1:320	
6.	1:640	
7.	Control	

Mix sera and antigen with toothpick or wooden applicator, starting with 1:640 dilution and working to the 1:20 dilution. Use a new applicator for each specimen. Shake slide slowly in rotary movement for 2 to 3 minutes. Read agglutination over artificial light above black background. Read reactions as 1, 2, 3 or 4 plus. Do not report 1 plus reactions but report all above 2 plus as positive agglutination.
Report the highest titer obtained (i.e.: Huddleson, Brucella 1:160).

B. **Tube Agglutination Test**—Inactivate serum at 55° C. for 30 minutes. Prepare dilutions of 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 or higher in small test tubes using 0.85 per cent saline. Combine equal quantities of the dilutions (0.5 c.c.) and killed suspensions of Brucella abortus (Dilute B. abortus concentrated suspension as supplied by U. S. Dept. of Agriculture, Bureau of Animal Industry 1:100 in 0.5 per cent phenol in 0.85 per cent sodium chloride), making total dilutions of 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280 or higher. Incubate at 48° to 52° C. for four hours and leave in refrigerator overnight. Tests may be incubated at 37° C. overnight, but reaction is usually not as marked at this temperature.
Report highest dilution in which definite agglutination occurs with B. abortus and when lower than 1:80 state that it is considered as a partial reaction. Report any other significant information in regard to the examination.

Interpretation:

Titer 1:160 or greater	Positive serological reaction for undulant fever. Suggest blood culture be taken at height of fever and submitted in special undulant fever outfit.

Titer 1:40 or 1:80	Reaction too weak to be diagnostic. Another specimen should be submitted to determine possible rise in titer.

VINCENT'S ANGINA
(Bacillus fusiformis and Borrelia vincenti)

METHOD:

A. Smear—Gram stain[33] preparations submitted on glass microscopic slides. Examine for fusiform bacilli and spirochetes having the morphology of those associated with Vincent's angina.

Report as "Spirochetes and fusiform bacilli having the morphology of those associated with Vincent's angina present (indicate the relative number) or absent."

B. Culture—It is as important to study a culture as well as a film when diagnosis of Vincent's angina is considered. Incubate cultures of exudate received on Loeffler's medium.[16] Treat cultures in the same manner as those submitted for diphtheria or septic sore throat. Examine culture by Gram's stain.[33]

Report as spirochetes and fusiform having the morphology of those associated with Vincent's angina "Present" or "Absent."

WEIL'S DISEASE—LEPTOSPIROSIS
(Leptospira icterohemorrhagiae)

A. Darkfield—(see under syphilis for technique)

Examine urine by darkfield. Examine for Leptospira (granular appearing fine spirochaetes with hooks at one or both ends). Organisms may be atypical in urine. Whether or not Leptospira are found, make guinea pig inoculation.

B. Animal Inoculation

Citrated blood may be used during the first week of the disease and urine from the 8th to 50th day or longer. Inject guinea pig (under 150 grams in weight) intraperitoneally with 2 to 3 c.c. of blood or urine. Observe for loss of weight and fever (may reach 103° F). The animal may die in 8 to 15 days with profound subcutaneous hemorrhage and marked

jaundice. Otherwise sacrifice animal after 15 days and examine a teased fragment of kidney and/or liver in saline by darkfield.

Note:
Observe strict aseptic precautions in the handling of all specimens for Leptospirosis.

METHOD:

C. Agglutination Test (Rapid)

Employing commercial antigen (Lederle), make the following serum dilutions: 1:10, 1:100 and 1:1000. Divide the surface of a microscope slide into four equal squares. Place one drop of each serum dilutions in a separate square and a drop of positive control serum in the fourth square. Add two drops of antigen to each drop of serum. Mix serum and antigen in each square with a wooden applicator. Tilt or rotate slide to continue mixing serum and antigen. Read agglutination before five minutes or before mixture begins to dry. Clumping of the antigen indicates positive serum. Report highest dilution giving agglutination (2 plus or higher).

Interpretation:

Agglutination in all dilutions (1:10, 1:100 and 1:1000) with L. icterohemorrhagiae	Positive serological reaction for Weil's Disease.
Titer 1:10 or 1:100	Reaction too weak to be diagnostic. Another specimen of blood should be submitted to determine possible rise in titer.

Note:
Initial agglutination in all dilutions or an increase in the agglutination titer during the course of the disease is usually considered to be significant.

D. Tube Agglutination Test

As the antigen employ a 4 to 6 day old L. icterohemorrhagiae culture[46] to which formalin (37 per cent) has been added (0.3 c.c. per 10 c.c. of culture). Employing the same technic as for other agglutination tests (see under enteric diseases) make serial dilutions of the patient's serum in Verwoort's medium[46] without rabbit serum. (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560 and control). The control tube contains Verwoort's medium[46] by itself. Transfer 0.15 c.c. of each dilution (working

from the highest dilution 1:2560 to the lowest dilution 1:10) to a series of small test tubes. Add 0.15 c.c. antigen to each of these tubes (final dilutions 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120 and control).

Incubate the tubes for 3 hours in a 37° C. waterbath. Employing a sterile platinum loop remove a drop from each dilution tube, place on a glass slide, with or without a coverglass, and examine for agglutination by darkfield under low or medium objective.

Report highest dilution giving agglutination (2 plus or higher).

For example: Agglutination L. icterohemorrhagiae 1:1280.

Interpretation: A specific agglutination reaction in a titer of 1:80 may be indicative of a past clinical or subclinical infection. A titer of 1:1280 or more is indicative of present or recent infection. Request "repeat" on low titers to determine possible rise in titer. Initial agglutination in all dilutions or an increase in the agglutination titer during the course of the disease is usually considered to be significant.

HEMATOLOGIC METHOD

METHOD

A. **Venous Blood Technic** (Osgood)—Prepare a month's supply of test tubes (⅝" x 6") measuring into each 0.1 c.c. of 2 per cent potassium oxalate (2 mgm) for each c.c. of blood to be taken. Evaporate to dryness in a drying oven. Keep tubes corked and in baskets labeled with the amount of blood for which they were prepared. Three to five c.c. of blood is sufficient for the routine hematologic examination. Using a 10 c.c. syringe have blood drawn from vein by usual technic. Place a small drop of blood on the edge of a clean and flamed microscope slide (for smear), remove needle from syringe, place measured volume of blood in oxalate tube and remainder of blood in sterile tube for Wassermann. Keep tube corked at all times when not in use and store in refrigerator (5 to 8° C.) till used. Shake oxalate tube vigorously by holding the test tube horizontally in the left hand and tapping the other end with the right hand. Make blood smear for differential in usual manner with smooth edge of another microscope slide and allowing smear to dry in air (do not heat or wave in air).

HEMATOLOGIC TEST PERFORMED WITH OXALATED VENOUS BLOOD
(after Osgood)

Method	Accurate if done within
1. Hemoglobin estimation	24 hours
2. Red cell count	24 hours
3. Platelet count	1 hour
4. Red cell volume	3 hours
5. Color index	24 hours
6. Volume index	3 hours
7. Saturation index	3 hours
8. Icterus index	4 hours
9. Van den Bergh test	4 hours
10. White cell count	24 hours
11. Making smear for differential count	1 hour
12. Peroxidase test	3 hours
13. Fragility test	3 hours
14. Sedimentation rate	3 hours

The time limits noted should be observed if the most accurate results are desired, although as a rule a slightly longer time will not introduce clinical error.

Thoroughly mix blood by shaking vigorously for 30 seconds by holding the test tube horizontally in the left hand and tapping the other end with the right hand immediately before samples are withdrawn from the test tube for any test. Samples should be taken directly from the test tube.

URINALYSIS
Routine Examination

PHYSICAL

A. **Color**—(urochrome)—amber (straw), colorless or some shade of amber.

B. **Odor**—(urinod)—aromatic, odorless, putrid, ammoniacal, fecal or of the preservative.

C. **Specific gravity**—the normal range is 1.014 to 1.024 with a urinometer.

D. **Transparency**
   1. Clear
   2. Cloudy
      a. With sediment.
      b. Without sediment.

E. **Reaction**—may be neutral or amphoteric—but that is rare.

1. Litmus
   a. Very faintly acid or alkaline.
   b. Faintly acid or alkaline.
   c. Markedly acid or alkaline.
2. Nitrozine paper gives an approximate pH.

F. **Note preservative added.**

MICROSCOPIC

A. Amorphous sediment

1. Phosphates—colorless—found in alkaline, occasionally in neutral and infrequently in acid urine.
   a. Insoluble when heated.
   b. Soluble in acetic acid.
2. Urates—some shade of yellow or brown—found in strongly acid urine.
   a. Soluble upon heating or when treated with alkali.
   b. Precipitate of uric acid is formed when hydrochloric acid or acetic acid is added.

B. Crystals

1. Calcium oxalate — colorless, octahedra — usually with a cross connecting the corners. Found in acid urine.
   a. Insoluble in alkalies and all organic acids (distinguished from uric acid and triple phosphate crystals).
   b. Soluble in dilute acids slowly and rapidly in strong mineral acids with difference.
2. Uric acid crystals rosette or cuboidal (yellowish crystals)
   a. Insoluble in hot water and acids.
   b. Soluble in alkalies.
3. Tyrosine
   a. Not soluble in glacial acetic, alcohol or ether.
   b. Soluble in hot water, alkali and dilute mineral acids.
4. Leucine
   a. Soluble in glacial acetic (distinguishes from tyrosine).
   b. Not soluble in hydrochloric acid or alcohol but soluble in alkali.

Tyrosine and leucine occur together, but there are usually more leucine crystals—greenish yellow sediment.

5. Cystine—found in normal urine (precipitated from acid urine).
   a. Insoluble in water (even boiling).
   b. Insoluble in acetic acid, alcohol and ether.
   c. Soluble in mineral acids and alkalies.
6. Crystalline phosphate—colorless, coffin lids or crude H-shaped crystals found in alkaline urine (dicalcium phosphate occasionally). Soluble in acetic acid. The crystals are usually magnesium ammonium phosphate which are colorless.
7. Acid ammonium urate crystals—("Thorn Apple")—some shade of yellow—found in alkaline urine.
   a. Soluble on heating, forms crystals again on cooling.
   b. Bubbles (ammonia) occur when treated with concentrated caustic alkali.

C. **Casts**—hyalin, light granular, dark granular, cellular, fatty, waxy, etc.

D. **Bacteria**

E. **Cylindroids**

F. **Red blood cells**

G. **White blood cells**

H. **Epithelium**

I. **Mucus**

J. **Spermatozoa**

CHEMICAL

A. **Reaction**

B. **Albumin** (Use any two tests)

1. Robert's test (contact test)—Place 5 c.c. Robert's reagent (1 part nitric acid plus 5 parts saturated solution of magnesium sulfate) in test tube, overlay surface with filtered 1 or 2 c.c. of urine. Yields a white zone at junction if "positive" for albumin.
2. Exton's test (contact test) — Exton's reagent[31] overlayed with urine yields a white zone at junction, if "positive" for albumin.

3. Purdy's test—Heat upper third of clear urine in tube. If cloudy solution appears at the top it may be either phosphates or albumin. Add a few drops 5% acetic acid—if cloudiness disappears it is phosphates—if cloudiness persists or intensifies it is albumin.

C. Sugar

1. Benedict's—Boil for 2 to 3 minutes 5 c.c. of Benedict's reagent plus 8 drops of urine—yields an opalescent green, yellow orange, orange brown or red, precipitate of cuprous oxide (trace), (1 or 2 plus), (3 or 4 plus).

D. Indican

1. Obermayer's—5 c.c. urine plus equal volume of Obermayer's reagent plus 2 c.c. of chloroform—shake well with finger over tube. (Iodides and thymol give the same blue color. Use sodium thiosulfate to clear up the color if urine has been preserved by thymol or patient is taking iodides).
2. Jaffe's—5 c.c. urine plus equal amount of concentrated HC1 plus 2 c.c. of chloroform. Shake well with finger over tube. Add few drops of aqueous solution of chlorinated lime—yields a blue color. Same interference as for Obermayer's.

E. Acetone

1. Legal's test—5 c.c. of urine plus a few crystals of sodium nitroprusside plus aqueous solution of sodium or potassium hydroxide yields a ruby red color. Add dilute acetic acid (5%)—if color becomes more intense it is acetone—if it disappears it is creatinine.

F. Diacetic Acid

1. Gerhart's (ferric chloride test)—to 5 c.c. add 10 c.c. aqueous ferric chloride solution until no more precipitate forms. A Bordeaux red color appears (may be due to diacetic acid or drugs). Boil 5 c.c. of urine and then add ferric chloride solution —no color discloses the presence of diacetic acid. Diacetic acid plus heat decomposes, forming acetone and produces no color (must be drugs if color appears).

G. Bile Pigment

1. Gmelin's test—Overlay 5 c.c. fuming nitric acid plus an equal amount of urine. At point of contact there is a play of colors (organic oxides—red, yellow, green, blue, violet).
2. Rosenbach's test—filter urine thru filter paper, add a few drops of concentrated nitric acid to filter paper. Note display of colors in center of paper.
3. Bile—Hay's surface tension test—Sprinkle flowers of sulfur over surface of urine—if particles of sulfur sink it is positive (bile lowers the surface tension of urine).

H. Occult blood

1. Benzidine—2 c.c. saturated solution of benzidine in glacial acetic acid plus 2 c.c. of urine plus 1 c.c. hydrogen peroxide yields a blue green color in 5 to 6 minutes if "Positive" for hemoglobin.
2. Guaiac—5 c.c. urine plus sufficient alcoholic solution of guaiac (soluble 1:60) to give a turbidity; add several drops of hydrogen peroxide—yields a blue color if "Positive" for hemoglobin.
3. Orthotoluidine—mix equal portions (1 c.c.) of reagent plus urine. Add hydrogen peroxide. A bluish color appears if "Positive" for hemoglobin. The reaction is slow.

Note:

Old pus, enzymes and certain milk give "False-positive" hemoglobin tests.

SANITATION
(Bacteriological and Chemical)

Water and Sewage Analyses—Perform in accordance with "Standard Methods for the Examination of Water and Sewage"—American Public Health Association. If necessary conduct Jackson sub-classification of B. coli group (A.P.H.A. 1912)

Eating Utensils (Bacteriological Examination)—The following is to be performed on all refrigerated swab specimens returned to the laboratory within 2 hours of the first sampling (each tube originally contained 10 c.c. of sterile Butterfield Buffer Solution[7]), mix tube contents well by holding top of tube firmly in left hand and rapidly striking the

bottom portion of the tube with the fingers of the right hand at least 25 times. Remove cotton stopper, squeeze cotton stopper to remove as much fluid as possible by rolling against inner surface of tube.

A. Inoculate surface of blood agar[7] plate by rolling swab over surface. Incubate at 37° C., examining after 24 and 48 hours' incubation for colony formation and hemolysis. Fish suspected colonies, stain by Gram's method[33] and examine. Report: Hemolytic bacteria "Present" or "Absent" and type. Example: Positive for hemolytic bacteria (streptococcus).

B. Plate 1 c.c. and 0.1 c.c. of the mixed saline solution in Neo-peptone yeast extract agar[19] and inoculate one lactose fermentation tube with 1 c.c. of the saline solution as per "The Standard Methods for the Examination of the Water & Sewage" of the American Public Health Association.

Report: The number of colonies appearing on a plate containing 1 c.c. of the saline solution, the number of organisms per utensil except for chopsticks for which it represents 2.5 of these items.

Based on the approximate area covered by swabbing, as indicated below, the following standards are recommended:

Glasses, cups, plates, bowls and knives — 500 colonies /utensil
Spoons and forks — 300 colonies /utensil
Chopsticks — 300 colonies /2.5 utensils

Types of utensil	Approx. area covered by swab per utensil (sq. in.)	No. of specimens constituting sample	Total area swabbed (sq. in.)	**Standard** colonies per 1 ml. plate
Glasses	9	10	90	500
Cups	9	10	90	500
Plates	9	10	90	500
Bowls	9	10	90	500
Knives	9	10	90	500
Spoons	5 to 6	10	60	300
Forks	5 to 6	10	50	300
Chopsticks	2	25	50	300

BOARD OF HEALTH
TERRITORY OF HAWAII

WATER ANALYSIS

Laboratory No. .....
Turbidity .....
Sample of ..... pH .....
From ..... NO_2 (ppm) .....
Collected .....Time.....m. NaCl (gr/G) .....
Examined .....Time.....m. Residual Cl_2 .....

STANDARD PLATE COUNT: (a)..... (b) ..... Average.....

PRESUMPTIVE TEST: Lactose peptone broth. If other broth is used, specify.

(Amount Tested)	24 hr. gas, %		48 hr. gas, %	
0.0001 c.c.	.....	.....	.....	.....
0.001 c.c.	.....	.....	.....	.....
0.01 c.c.	.....	.....	.....	.....
0.1 c.c.	.....	.....	.....	.....
1 c.c.	.....	.....	.....	.....
10 c.c.	.....	.....	.....	.....
100 c.c.	.....	.....	.....	.....
	.....	.....	.....	.....
	.....		.....	

CONFIRMED TEST: (Typical) (Intermediate) (Atypical)
on.....agar.....% gas in.....broth.

IDENTIFICATION:	1	2	3	4
Lactose	.....	.....	.....	.....
Gram stain	.....	.....	.....	.....
Motility	.....	.....	.....	.....
Indol	.....	.....	.....	.....
Methyl red	.....	.....	.....	.....
Voges Proskauer	.....	.....	.....	.....
Citrate	.....	.....	.....	.....
Dulcitol	.....	.....	.....	.....
Sucrose	.....	.....	.....	.....
Salicin	.....	.....	.....	.....
.....	.....	.....	.....	.....

Organisms of the Coliform Group

1. .....
2. .....
3. .....
4. .....

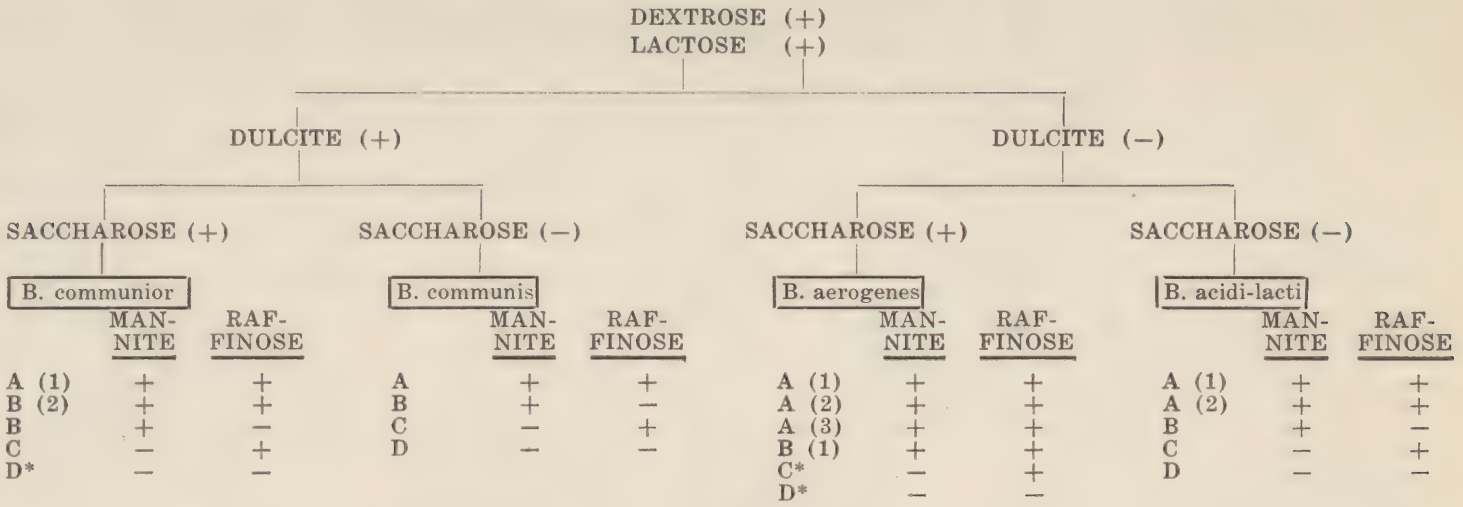
MOST PROBABLE NUMBER coliform per 100 c.c. sample:.....

RAPID CHEMICAL TESTS:

Pb	CN	Aldehyde .....
Hg	Alkaloid	Fluoride .....
As	Phenol	Glucoside .....
Ba	Thallium	....

REMARKS: .....

JACKSON CLASSIFICATION OF COLON GROUP



B. communior	MANNITE	RAFFINOSE
A (1)	+	+
B (2)	+	+
B	+	−
C	−	+
D*	−	−

B. communis	MANNITE	RAFFINOSE
A	+	+
B	+	−
C	−	+
D	−	−

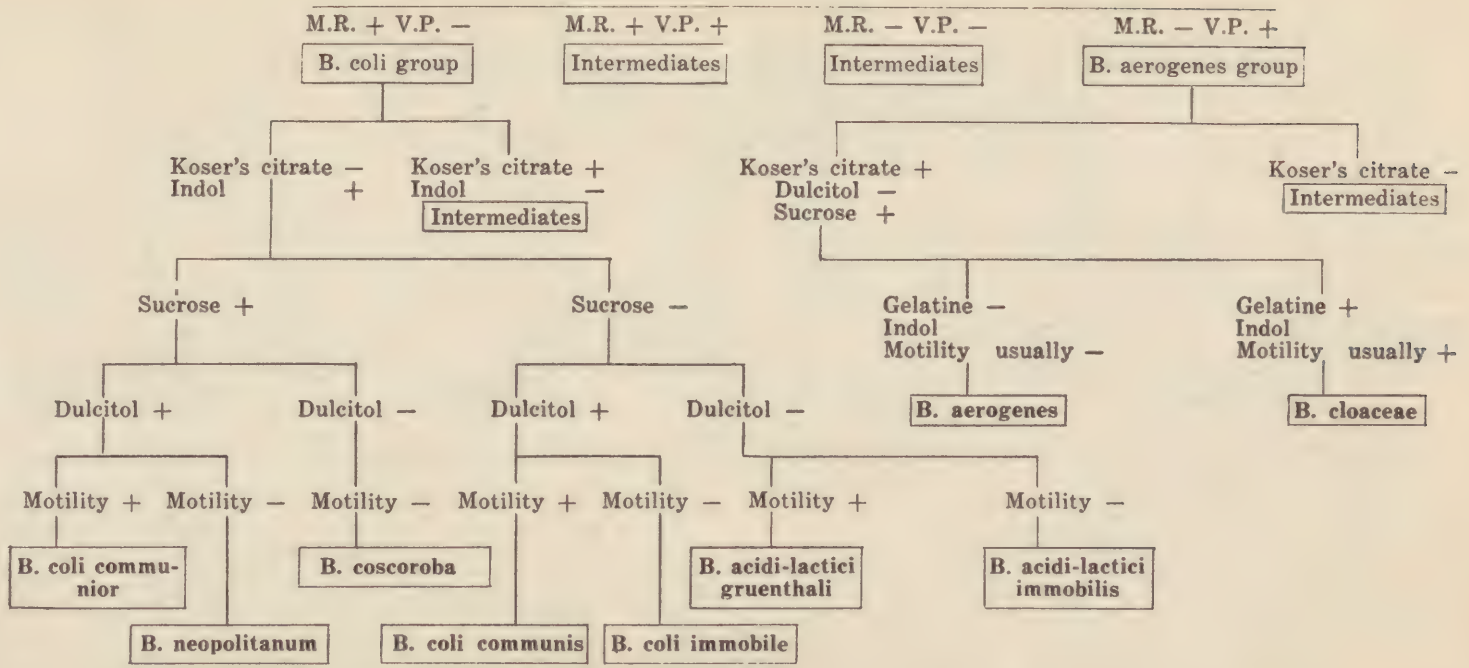
B. aerogenes	MANNITE	RAFFINOSE
A (1)	+	+
A (2)	+	+
A (3)	+	+
B (1)	+	+
C*	−	+
D*	−	−

B. acidi-lacti	MANNITE	RAFFINOSE
A (1)	+	+
A (2)	+	+
B	+	−
C	−	+
D	−	−

A, B, C, D, etc. ..... Varieties
\+ ..... Positive reaction (Acid and Gas)
− ..... Negative reaction
* ..... Unknown variety
1, 2, 3 ..... Subvarieties

KEY TO THE CLASSIFICATION OF COLIFORM ORGANISMS

(Gram-negative, non-sporing, lactose-fermenting (AG), aerobic bacilli)



Note: + = fermentation with the production of acid and gas.
– = no fermentation with the production of acid and gas.
In our routine work, classification of B. aerogenes and B. cloaceae is based on indol and motility. Gelatine is deleted because liquefaction is generally very slow (7 to 10 days).
Motility is determined either microscopically or by the use of semisolid agar or both.

Reference: 1. Levine, Max. 1921. Bacteria Fermenting Lactose and Their Significance in Water Analysis. Iowa State College of Agr. & Mech. Arts. Bulletin 62, 127 pp. Official Publication Vol. XX, No. 31, 127 pp.
2. Thresh, J. C., J. F. Beale and E. V. Suckling. 1933. The Examination of Waters and Water Supplies. 4th Ed. P. Blakiston's Son & Co. Inc. Philadelphia, pp. 436-60.

RELATIVE PROPORTIONS OF SOME OF THE MEMBERS OF THE COLIFORM GROUP FOUND IN HUMAN AND ANIMAL FECES

(PER CENT)

Lactose Fermenting Bacilli	MacConkey			Clemesha		Group Representative Organism
	Human	Cowdung	Animal	Human	Cowdung	
B. acidi lactici B. grunthal B. levans	34	16.6	15	53.2	49.3	B. acidi lactici
B. coli communis	38	25.0	36	17.4	9.4	B. coli communis
B. coli communior B. oxytocus perniciosus B. neopolitanus	15	47.9	31	6.8	16.0	B. coli communior
B. lactis aerogenes B. cloacae B. coscoroba	12	12.5	18	22.2	35.3	B. lactis aerogenes

Ref.: Thresh, J. C., J. F. Beale and E. V. Suckling. 1933. The Examination of Waters and Water Supplies. 4th ed. P. Blakiston's Son & Co. Inc. Philadelphia, p. 439, Tables I & II.

DISTRIBUTION OF COLIFORM ORGANISMS FROM DIFFERENT SOURCES AMONG THE VARIOUS SPECIES AND VARIETIES

		B. cloacae	B. aerogenes	B. communior	B. neopolitanus	B. coscoroba	B. coli communis	B. coli immobilis	B. acidi-lactici gruenthali	B. acidi-lactici immobili	Total
Soil	No.	88	54	26	0	0	2	0	7	0	177
	%	49.7	30.5	14.7			1.1		4.0		
Horse	No.	0	0	15	0	0	4	0	0	0	19
	%			79.0			21.0				
Sheep	No.	0	0	16	0	5	1	0	0	0	22
	%			72.8			4.5				
Cow	No.	0	0	6	4	0	9	0	1	0	20
	%			30.0	20.0		45.0		5.0		
Pig.	No.	0	0	9	0	1	11	1	9	0	31
	%			29.0		3.2	35.6	3.2	29.0		
Sewage	No.	1	8	3	3	2	1	12	2	7	39
	%	2.6	20.5	7.7	7.7	5.1	2.6	30.8	5.1	17.9	
Man	No.	0	0	2	0	1	5	5	1	11	25
	%			8.0		4.0	20.0	20.0	4.0	44.0	
Total		89	62	77	7	9	33	18	20	18	333

Max Levine, 1921. Bacteria Fermenting Lactose and Their Significance in Water Analysis. Bul. 62, Eng. Exp. Sta., Ames, Iowa, p. 88.

Milk and Dairy Products—Microbiological, Bio-assay and Chemical analyses are made in accordance with: **"Standard Methods for the Examination of Dairy Products"** published by the American Public Health Association.

Bottled Beverages—Perform examinations in accordance with the methods prescribed by the **"Committee on Microbiological Examination of Foods"** of the American Public Health Association as found in the **American Public Health Association Year Book 1941-1942, pages** 96 to 100.

Chemical Analysis—Perform in accordance with latest editions of the **"Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists,"** the **"United States Pharmacoepia"** and the **"National Formulary."**

POI

METHOD:

A. **Total Solids**—Weigh a flat bottom dish of not less than 5 cm. diameter. Spread approximately 3 to 5 grams of poi over inner bottom surface of dish. Evaporate to constant weight in oven at 100° C. (3½ hours to 12 hours). Cool in a dessicator and weigh quickly to avoid absorption of moisture.

Report as percentage of total solids.

Example:

1. Weight of evaporating dish.....19.75 grams
2. Weight of evaporating dish and poi .....24.26 grams
3. Weight of evaporating dish and dried poi .....20.11 grams

Difference between (1) and (2)—
4.51 grams net weight of sample
Difference between (1) and (3)—
1.36 grams net weight of sample
Percentage $\frac{1.36}{4.51}$ of 100% =30.15% total solids

Note:

Poi with 15% total solids is very thin and flows like "thick soup." It may be **"ready-mixed"** poi or poi prepared for serving, as is sold in restaurants. However, ready-mixed poi usually runs from 17 to 20 per cent in total solids.

VI. Culture Media, Reagents and Stains

pH INDICATOR SOLUTIONS FOR TITRATION PURPOSES

Indicator	pH Range		Formula
Methyl red	red 4.2	yellow 6.3	0.2 gm. in 60 c.c. alcohol plus 40 c.c. distilled water
Brom cresol purple	yellow 5.2	purple 6.8	Grind 0.1 gm. in 18.5 c.c. 0.01 normal NaOH; dilute to 250 c.c. with alcohol
Brom thymol blue	yellow 6.1	blue 7.6	Grind 0.1 gm. in 16 c.c. 0.01 normal NaOH; dilute to 250 c.c. with alcohol
Phenol red	yellow 6.8	red 8.4	Grind 0.1 gm. with 28.2 c.c. 0.01 normal NaOH; dilute to 250 c.c. with alcohol

pH INDICATOR SOLUTIONS FOR CULTURE MEDIA

(unless otherwise specified, use 1 c.c. per 1000 c.c. of medium)

Indicator	pH Range		Formula
Brom cresol purple	yellow 5.2	purple 6.8	1.6 gm. qsd to 100 c.c. with alcohol
Brom thymol blue	yellow 6.0	blue 7.6	1.6 gm. qsd to 100 c.c. with alcohol
Phenol red	yellow 6.8	red 8.4	1.6 gm. qsd to 100 c.c. with alcohol
Azolitmin	red Acid	blue Alkaline	1 gm. in 80 c.c. hot distilled water, add 20 c.c. alcohol, mix
Andrade	pink Acid Colorless or yellow Alkaline	colorless pH 7.2	Dissolve 0.5 gm. acid fuchsin in 100 c.c. distilled water. Add normal sodium hydroxide until color changes from red to orange or yellow (about 16 c.c. of normal sodium hydroxide are usually required). Filter and sterilize in autoclave at 15 pound pressure for 20 minutes.

1. ASCITIC AGAR

To 1000 c.c. of liquified sterile agar[20] (pH 7.4—7.8) cooled to 50° C., add 200 c.c. of sterile ascitic fluid previously warmed to about 50° C. Mix well by rotation.

2. BEEF OR VEAL INFUSION BOUILLON

Minced lean beef or veal ..... 500 gm.
Distilled water .....1000 c.c.

Mix well and place in a shallow dish in a refrigerator to soak for about 24 hours. Skim off fat, filter through cheese-cloth and express juice contained in the meat. Boil for one-half to one hour or until the fluid is clear. Strain through cheese-cloth and filter.

ADD: Peptone .....10 gm.
Sodium chloride (C.P.) ..... 5 gm.

Boil for 15 minutes. Bring to original volume (1000 c.c.). Adjust reaction to pH 7.0, filter, place in suitable containers (tubes or flasks) and autoclave at 15 pound pressure for 20 minutes.

3. BEEF-HEART BROTH

Ground beef heart (fresh, lean) ..... 500 gm.
Distilled water .....1000 c.c.

Mix well and place in shallow dish in a refrigerator and allow to soak overnight. Drain. Save juice and meat. To one part of beef juice add 2 parts nutrient broth adjusted to pH 7.6 with 2 Normal sodium carbonate, boil 10 minutes and recheck pH to 7.6.

Tube to form a column about 7 cm. high (about 15 c.c.) in a 6 x ¾ inch test tube with 2 cm. of ground beef heart in the bottom. Add about 0.5 cm. layer of vaseline. Sterilize one hour at 15 pound pressure.

4. BLOOD AGAR (5 per cent)

Blood, (horse, rabbit or human)
defibrinated or citrated and sterile.... 50 c.c.
Nutrient agar (pH 7.4) .....950 c.c.

Liquify nutrient agar and cool to 50° C.; aseptically add the blood, previously warmed to 50° C., to the agar and mix well. Aseptically tube, slant or pour plates. Allow to harden.

5. BLOOD BROTH (Avery)

To veal infusion broth[2] ..... 100 c.c.
add citrated sheep, rabbit or human blood ..... 5 c.c.
and sterile glucose solution (1 c.c. of sterile 10% glucose) to make 0.1 per cent. Incubate tubes overnight to check sterility.

6. BORDET-GENGOU POTATO BLOOD AGAR

A. Boil until soft
peeled sliced potatoes ..... 500 gm.
in glycerin ..... 40 c.c.
and distilled water ..... 1000 c.c.

Make up to volume (1000 c.c.), strain through gauze and allow to stand for sedimentation. Siphon off supernatant liquid.

B. To clear potato extract (A) ..... 500 c.c.
add sodium chloride solution (0.75 per cent aqueous) ..... 1500 c.c.
agar (to make 2.5 per cent) ..... 50 gm.

Allow mixture to stand for 15 minutes to saturate agar. Heat until agar is dissolved and dispense in 100 c.c. quantities. It is not necessary to adjust the reaction which is usually somewhat less than pH 6.5. Autoclave at 15 pounds for 25 minutes.

C. To 100 c.c. of melted base (B) at 45° C. add 20 c.c. of citrated blood (not over 72 hours old) (final concentration 16 2/3 per cent). Mix sheep, horse, rabbit or human blood with base by whirling and pour into petri dishes. Check sterility by incubating at 37° C. for 24 hours. Inoculate one plate with Hemophilus pertussis to check growth-promoting, colony appearance and characteristic hemolytic zone. Plates may be used as long as they remain moist and red—usually about 2 weeks, when kept in the refrigerator.

7. BUTTERFIELD BUFFER SOLUTION (For swabbing of eating utensils)

A. Phosphate buffer solution:
Dissolve KH_2PO_4 ..... 34.0 grams
in distilled water ..... 500 c.c.

Adjust to reaction to pH 7.2 with one molar sodium hydroxide solution and make up to one liter with distilled water.

Sterilize by autoclaving at 15 pounds for 20 minutes. Distribute measured quantities aseptically into sterile tubes.

8. CHOCOLATE AGAR
(pH 7.6—7.8)

A. Dissolve with the aid of heat:

Agar, Bacto Proteose #3 ..... 45 gm.
in distilled water .....500 c.c.
Q. S.

Mix well, dispense accurately measured quantities in suitable containers and autoclave at 15 pounds for 20 minutes.

B. Dissolve:

Hemoglobin, Bacto ..... 10 gm.
in distilled water (heated to 50° C.)....500 c.c.

When solution is nearly complete, filter through coarse, moistened cheese-cloth to remove undissolved particles. Dispense accurately measured quantities in suitable containers (same volume as agar) and autoclave at 15 pounds for 20 minutes.

C. Dissolve:

Para aminobenzoic acid ..... 10 gm.
in distilled water .....100 c.c.

Sterilize in autoclave at 7 pound pressure for 30 minutes.

D. Dissolve:

Cysteine monohydrochloride .....10 gm.
in distilled water .....50 c.c.

Sterilize in autoclave at 7 pound pressure for 30 minutes.

After cooling both the agar (A) and hemoglobin solution (B) to between 50° and 60° C., aseptically add 5 c.c. para aminobenzoic solution (C) and 2.5 c.c. cysteine monohydrochloride solution (B) to melted agar (A). Mix agar (A, C, D) and hemoglobin (B) in equal quantities under aseptic conditions. Avoid air bubbles* and pour into sterile petri dishes (about 20 c.c. each). Allow to solidify and store in refrigerator in inverted position.

*Although care against an abundance of air bubbles is taken, some bubbles often appear in the media. These may be removed by flaming the surface of the media with a Bunsen burner before the media has solidified (the bubbles burst due to heat expanding the air therein contained).

9. DIEUDONNE'S MEDIUM

Mix defibrinated sheep blood ..... 15 c.c.
with normal potassium hydroxide ..... 15 c.c.
Heat 30 minutes at 100° C. (keeps several months)
Add nutrient agar (50° C.) ..... 70 c.c.

Pour plates and dry inverted, without covers, in incubator at 37° C. for 24 hours to permit ammonia to escape.

10. DUNHAM'S PEPTONE SOLUTION

Dissolve

Peptone ..... 10 gm.
Sodium chloride ..... 5 gm.
in water ..... 1000 c.c.

by boiling for 15 minutes. Make up for evaporation, filter, tube and sterilize by autoclaving at 15 pounds for 20 minutes.

11. ENDO AGAR (Levine)*
(pH 7.6—8.0)
(For colon-typhoid differentiation)

A. Dissolve

Lactose ..... 10 gm.
Dibasic potassium phosphate ..... 3.5 gm.
in hot liquified nutrient agar ..... 960 c.c.

B. Dissolve

Sodium carbonate (anhydrous) ..... 1 gm.
in distilled water ..... 10 c.c.

C. Dissolve

Basic fuchsin (90% dye content) ..... 0.5 gm.
in alcohol (95%) ..... 5 c.c.

D. Dissolve

Sodium bisulfite ..... 2.5 gm.
in distilled water ..... 25 c.c.

Add the sodium carbonate solution, the fuchsin solution and the sodium bisulfite solution to the nutrient agar solution, mixing well after each addition. Place in suitable containers and sterilize at 15 pound pressure for 20 minutes.

*Prepare freshly, as needed—deteriorates upon standing, especially if exposed to light.

12. EOSIN METHYLENE BLUE AGAR (Levine)
(For Colon-Aerogenes differentiation)

Dissolve with the aid of heat

A. Peptone ..... 10 gm.
Dibasic potassium phosphate ..... 2 gm.
Agar ..... 1000 c.c.
in distilled water

Replace the water lost by heating (q.s. to 1000 c.c.). Place 100 c.c. quantities in flasks and sterilize by heating in autoclave at 15 pounds for 20 minutes.

B. Just prior to use, liquify the medium with the aid of heat and to each flask containing 100 c.c. aseptically add

Lactose (sterile 20% aqueous solution) .. 5 c.c.
Eosin Y (85% dye content—2 per cent sterile aqueous solution) ..... 2 c.c.
Methylene Blue (90% dye content—sterile 0.5 per cent aqueous solution) .. 2 c.c.

13. KRACKE MEDIUM

A. Infuse

Finely ground heart muscle ..... 500 gm.
with distilled water ..... 1000 c.c.

Mix well. Allow to soak in refrigerator for about 24 hours. Press infusion through cheese-cloth and express juice contained in the meat. Heat to boiling point without constant stirring. Pass infusion through fine mesh cheese-cloth or allow to settle and decant.

B. Macerate

Brain tissue (bovine) ..... 250 gm.
in distilled water ..... 500 c.c.

Strain the infusion through a double layer of cheese-cloth. Heat filtrate slowly to boiling point with constant stirring (this process coagulates the particles of brain tissue and leaves them in a state of fine suspension). Do not filter.

C. Mix

Heat muscle infusion (A) ..... 750 c.c.
Brain tissue infusion (B) ..... 250 c.c.
Sodium citrate ..... 1 gm.
Dextrose ..... 10 gm.
Peptone ..... 10 gm.
Dibasic sodium phosphate ..... 2 gm.
Sodium chloride ..... 4 gm.

Heat to effect solution, adjust reaction to pH 7.4. Dispense (about 50 c.c.) in containers (75 to 100 c.c.), thus allowing

at least 1/5 of the space for patient's blood. Autoclave at 15 pounds for 20 minutes.

14. LACTOSE BROTH
(For APHA water analysis)

Beef extract	3 gm.
Peptone	5 gm.
Lactose	5 gm.
Distilled water q.s.	1000 c.c.

Dissolve ingredients in water, boil a few minutes, make up to original volume (1000 c.c.). Adjust pH 6.8, filter, tube and autoclave at 15 pound pressure for 15 minutes.

15. LITMUS MILK

Add a sufficient quantity of an aqueous solution of azolitmin (see indicators) to fresh skim milk to produce a violet-blue color. Tube and sterilize in Arnold steam sterilizer for one-half hour on three successive days or at 7 pounds for 45 minutes in the autoclave.

16. LOEFFLER'S BLOOD-SERUM MEDIA

Blood serum (horse, beef or sheep)	300 parts
Glucose bouillon (nutrient broth[18] containing 1% glucose pH 6.8-7.0)	100 parts

Place in test tubes, slant in racks. Place in cold autoclave, bringing pressure gradually to 15 pounds with temperature around 80° C. (air not exhausted). Release air and replace with steam without changing pressure, gradually. When operating at 121° C. and 15 pound pressure, continue for 15 minutes—sterilize. Incubate slants at 37° C. overnight and check for sterility.

17. MacCONKEY AGAR

A. Dissolve with aid of heat

Peptone	17 gm.
Proteose peptone	3 gm.
Lactose	10 gm.
Bile salts (Bacto #3)	1.5 gm.
Sodium chloride	5 gm.
Agar	20 gm.
in distilled water	1000 c.c.

B. Dissolve

Neutral red	0.03 gm.
in alcohol (95%)	5 c.c.

C. Dissolve

Crystal violet (certified)	0.001 gm.
in alcohol (95%)	5 c.c.

Add neutral red solution (B) and crystal violet solution (C) to agar solution (A). Make up to volume (1000 c.c.) and mix well. Dispense in suitable containers and autoclave at 15 pound pressure for 20 minutes. About 20 c.c. of medium should be poured into plates when the agar has cooled to about 50° C.

18. MEAT EXTRACT BOUILLON (Nutrient Broth)

Make a paste of

Peptone	10 gm.
Sodium chloride (c.p.)	5 gm.
Dissolve in distilled water	1000 c.c.
Add beef extract (Liebig type)	3 gm.

Bring to boil and boil slowly for 15 to 20 minutes. Make up for evaporation by the addition of distilled water to original volume (1000 c.c.), adjust reaction (pH 7.0), filter, dispense and sterilize in the autoclave at 15 pound pressure for 20 minutes.

19. NEOPEPTONE YEAST EXTRACT AGAR

Dissolve with the aid of heat

Neopeptone (Bacto-Difco)	10.0 gm.
Yeast extract (Bacto-Difco)	5.0 gm.
Dextrose	0.5 gm.
Sodium chloride	5.0 gm.
Agar	15.0 gm.
in distilled water	1000 c.c.

Make up to volume (1000 c.c.) and adjust reaction to pH 7.5. Dispense in suitable containers and sterilize in the autoclave at 15 pound pressure for 15 minutes.

20. NUTRIENT AGAR

Bouillon[18]	1000 c.c.
Agar	15 gm.
	(or from 1 to 3 per cent.)

Boil slowly and stir frequently until the agar is dissolved. Adjust reaction to pH 7.0 (pH 7.4 for blood agar) and filter through a moderately thick layer of absorbent cotton con-

tained in a funnel (it is advisable to pass hot water through the cotton first, so as to warm the funnel and flask). Dispense and sterilize in the autoclave at 15 pound pressure for 20 minutes.

21. NUTRIENT GELATIN

Dissolve with gentle heat

Gelatin ..... 200 gm.
in nutrient broth ..... 1000 c.c.

Adjust reaction to pH 7.0. Cool to 50° C. Add the white of one egg, bring to boil and filter through gauze and cotton. Dispense in adequate containers. Sterilize in the autoclave at 15 pound pressure for 20 minutes.

Note:

Preferably tubes should never be allowed to liquify, except by enzyme action, necessitating incubation at 20 to 25° C. for two or more days. Gelatin properly made and not overheated should not melt much below 28° C. If necessary incubate at 37° C. and test by icing tube, which should solidify in the absence of enzyme (gelatinose).

22. PETRAGNANI'S MEDIUM (Mac Nabb)

In a liter flask, mix

Potato starch ..... 6 gm.
Milk ..... 160 c.c.
and one peeled and grated potato (size of an egg)

Shake vigorously in boiling water till a colloid dough is formed. Leave in boiling water for one hour. Cool to 60° C. Add four eggs and one yolk (the yolks of five eggs and the white of four eggs). Add 12 c.c. glycerin (or bovine bile, when bovine strains are suspected). Add 10 c.c. of a 2 per cent aqueous malachite green solution. Shake well. Filter through gauze, dispense and inspissate as for Loeffler's media.[16]

23. RUSSELL'S DOUBLE SUGAR AGAR

Dissolve

Lactose ..... 10 gm.
Dextrose ..... 10 gm.
in hot liquified nutrient agar[20] pH 7.2-7.6 ..... 1000 c.c.
Add phenol red indicator (0.02 per cent aqueous) ..... 1.5 c.c.

Mix thoroughly, tube and sterilize in the autoclave at 10 pound pressure for 30 minutes. Allow tubes to solidify in slanting position.

24. SABOURAD'S AGAR
(Weidman's modification)

Dissolve with the aid of heat

Peptone	10 gm.
Agar	18 gm.
Dextrose	40 gm.
in distilled water	1000 c.c.

Filter through cheese-cloth and sterilize at 10 pound pressure for 30 minutes.

25. SEMI-SOLID AGAR

Proceed as directed under nutrient agar[20] using 2 gm. of agar for each 1000 c.c. of nutrient broth. Adjust reaction to pH 8.0 to 8.2. Dispense in tubes so that depth of medium is at least 6 to 7 cm. Autoclave at 15 pounds for 20 minutes.

26. S. S. AGAR

A. Dissolve with aid of heat

Beef extract	5 gm.
Proteose peptone	5 gm.
Lactose	10 gm.
Bile salts (Bacto #3)	8.5 gm.
Sodium citrate	8.5 gm.
Sodium thiosulfate	8.5 gm.
Ferric citrate	1.0 gm.
Agar	17 gm.
in distilled water	1000 c.c.

Adjust reaction of medium to pH 7.2.

B. Dissolve neutral red ..... 0.025 gm.
in alcohol (95%) ..... 5 c.c.

C. Dissolve brilliant green (certified) ..... 0.0033 gm.
in alcohol (95%) ..... 5 c.c.

Add neutral red solution (B) and brilliant green solution (C) to agar solution (A) and make up to volume (1000 c.c.). Reheat medium to boiling—**DO NOT STERILIZE.** Pour about 20 c.c. into petri dishes and allow to dry for about 2 hours with covers partially removed.

27. TELLURITE BLOOD MEDIUM

A. Add 1 per cent potassium tellurite to defibrinated beef blood (or use Bacto tellurite blood solution)

B. To proteose #3 agar ..... 100 c.c.
aseptically add tellurite blood
solution (A) ..... 5 c.c.

Heat mixture to 75° to 80° C. until it takes appearance of chocolate agar. Cool to 50° C., pour into sterile petri dishes and allow to solidify.

REAGENTS AND STAINS

28. BENEDICT'S "QUALITATIVE" REAGENT

Copper sulphate ..... 17.3 gm.
Sodium or potassium citrate, c.p. .... 173.0 gm.
Crystalline sodium carbonate ..... 200.0 gm.
Distilled water, q.s. ..... 1000.0 gm.

Into a pyrex beaker place the requisite amount of citrate and add crystalline carbonate. Dissolve these ingredients in 700 c.c. distilled water by the aid of heat. Filter if necessary. Dissolve the copper in 100 c.c. water and pour the copper solution into the above alkaline solution, stirring constantly. Allow to cool and bring the volume up to 1000 c.c.

29. CARBOL-XYLOL

By volume:

Phenol crystals 1/3
Xylol 2/3

30. CHROMIC ACID CLEANING SOLUTION FOR GLASSWARE

Potassium dichromate ..... 60 gm.
Distilled water ..... 300 c.c.
Sulfuric acid (concentrated) 460 gm. (252 c.c.)

31. EXTON'S REAGENT

Sodium sulfate ..... 200 gm.
Sulfosalicylic acid ..... 50 gm.
Distilled water, q.s. ad. ..... 1000 c.c.

Dissolve sodium sulfate in 700 c.c. water with heat; allow to cool. Dissolve the acid without heat and dilute to 1000 c.c.

32. GIEMSA'S STAIN

A. Dissolve Giemsa stain (certified) 0.5 gm. in 33 c.c. of glycerin c.p. (neutral) at 55° to 60° C. for 1½ to 2 hours. To this add methyl alcohol (absolute, acetone free, neutral) 33 c.c.

B. Buffer solution (pH 7.0 to 7.2)

Dissolve mono-potassium phosphate .....5.33 gm.
and disodium phosphate .....14.824 gm.
in a liter of distilled water employing Brom thymol blue as indicator.

METHOD:

For use add one drop of dye solution (a) to 1 c.c. of buffer solution (b) (pH 7.0 to 7.2); or 0.3 c.c. of dye solution (a) to 15 c.c. of buffer solution (b). Cover smear of set slides on end in stain solution and leave for 45 minutes. At end of this time remove slides and set them in neutral distilled water for 3 to 5 minutes. Drain dry and examine.

33. GRAM'S STAIN (Hucker's modification)

A. **Gentian Violet**

1. Dissolve crystal violet ..... 4 gm.
(certified—85% dye content)
in ethyl alcohol (95%) ..... 20 c.c.
2. Dissolve ammonium oxalate .....0.8 gm.
in distilled water ..... 80 c.c.

Dilute stock solution of crystal violet (1) 1:10 with distilled water.

Mix one part of the diluted crystal violet solution with 4 parts of ammonium oxalate solution (2).

B. **Gram's Iodine Solution** (10X formula) (Stock solution)

Employing a mortar and pestle,
dissolve iodine .....10 gm.
and potassium iodide .....20 gm.
in a few c.c. of distilled water.

When complete solution has taken place, add distilled water to total 300 c.c. (including few c.c. used for initial solution). This is a stock solution which will keep for many months if kept in a well-stoppered bottle in a dark place at room temperature.

For use, dilute the stock solution 1:10 with distilled water (10 c.c. of 10X iodine solution and 90 c.c. of distilled water). This solution deteriorates on standing in the laboratory and should be prepared freshly at least every two weeks.

C. **Counterstain** (Stock solution)

Dissolve Safranin (certified) ..... 2.5 gm.
in ethyl alcohol (95%) .....1000 c.c.

For use, mix safranin stock solution (1) with distilled water 1:11 (safranin stock solution 10 c.c. with distilled water 100 c.c.).

GRAM'S STAINING PROCEDURE:

Stain smear one minute with crystal violet solution (stains everything blue or violet). Wash in running tap water (to wash off excess stain). Apply iodine solution for one minute (mordant). Pour off excess iodine solution. Wash with 95% alcohol drop by drop until no more color comes away (approximately 30 seconds). Wash in running tap water (to wash off alcohol). Counterstain with safranin for 10 seconds. Wash in water. Drain and dry without blotting. Those bacteria which retain the gentian violet color after this treatment are known as "gram positive" bacteria. Those microorganisms that lose their color and take the counterstain are called "gram negative."

34. IMMERSION OIL

Make 18 c.c. of a-bromonaphthalene up to 100 c.c. with Nujol (mineral oil). This immersion oil is optically satisfactory for microscope use, is not sticky and will not dry. It gives a refractive index equivalent to that of oil of cedar used for microscopic work (refractive index 1.515 at 18° C.).

35. IODINE-EOSIN STAIN

(Kofoid's modification of Donaldson's stain)

Mix equal parts of eosin (saturated solution in saline (0.85%) and 5% potassium iodide in saline (0.85%) saturated with iodine.

NOTE:

This mixture should be used when not more than 12 hours old.

36. IRON-ALUM

Dissolve:

Ferric ammonium sulphate crystals (clear and purple or lavender) ..... 2 gm.
in distilled water—q.s. .....100 c.c.

37. IRON HEMATOXYLIN

Solution A—Dissolve Hematoxylin ..... 1 gm.
in ethyl alcohol (absolute) ....100 c.c.
Place in bottle, cork and permit to stand in the light for one month or longer. (Hematoxylin must be properly ripened to secure satisfactory results.)

Solution B—Dissolve Ferric ammonium sulfate (violet crystals) ..... 8 gm.
Ferrous ammonium sulfate ..... 8 gm.
Distilled water .....40 c.c.
When dissolved add solution to 1000 c.c. dis-

tilled water which has been acidified with 10 c.c. hydrochloric acid (USP). Mix equal portions of solutions A and B immediately before using.

38. METHYLENE BLUE

Dissolve Methylene blue (certified) ..... 0.3 gm.
in ethyl alcohol (95%) ..... 30.0 c.c.
When dissolved add distilled water ....100.0 c.c.

Stain smears for 10 seconds to one minute. Wash with water and drain and/or blot dry.

39. MOUNTING MEDIUM

Dissolve Canada turpentine (Canada Balsam, Balsam of Fir), dried to remove the volatile oil, in a sufficient quantity of xylene. The solution may be thinned by adding xylene or thickened by evaporation.

40. OBERMAYER'S REAGENT

Add 2 grams of ferric chloride to one liter of hydrochloric acid (specific gravity, 1.19).

41. PEPSIN DIGESTION SOLUTION

Dissolve:

Pepsin (granulated) USP .....2.5 gm.
Hydrochloric acid (USP or CP) .....10 c.c.
Sodium chloride (USP or CP) .....2.0 gm.
in 1000 c.c. distilled water

42. PERRIN'S STAIN

Distilled water .....10 c.c.
Commercial formalin ..... 1 c.c.
Acetic acid ..... 1 c.c.
Ziehl's fuchsin ..... 4 c.c.
The stain will keep well for a month.

43. SCHAUDINN'S FIXATIVE

Stock Solution: (keeps indefinitely)

Add saturated (20% at room temperature) solution of mercuric bichloride in physiological saline .....2 parts
to ethyl alcohol (95%) .....1 part

For use:

to stock solution ..... 96 c.c.
add glacial acetic acid (USP) ..... 4 c.c.

NOTE:

This final solution should be changed frequently.

44. STOOL PRESERVATIVE (pH 7.2)

Glycerin ..... 30 c.c.
0.6 per cent saline ..... 70 c.c.
Dibasic potassium phosphate
(anhydrous) ..... 3.1 gm.
Monopotassium phosphate (anhydrous 1 gm.

If necessary adjust the pH to 7.2 with normal hydrochloric acid or normal sodium hydroxide. Two parts of this preservative, when added to one part of feces, will satisfactorily preserve bacilli of enteric diseases up to four days.

45. TOLUIDIN BLUE STAIN
(Ponder's)

Dissolve:

Toluidine blue (certified) ..... 0.02 gm.
Glacial acetic acid (USP) ..... 1 c.c.
When dissolved, add to ethyl alcohol
(absolute) ..... 2 c.c.
With distilled water make volume up to 100 c.c.

Stain fixed smears 8 to 10 minutes, wash with water, drain and/or blot dry and examine. Diphtheria appear as blue rods with red granules.

46. VERWOORT'S MEDIUM
(Schuffner modification)

A. **Peptone Solution**

Dissolve by boiling:

Proteose #3 Peptone (Difco) ..... 1.5 gm.
in distilled water ..... 1500 c.c.

B. **Phosphate Buffer Mixture**

Dissolve:

Potassium phosphate (monobasic) .... 0.35 gm.
Sodium phosphate (dibasic) ..... 1.33 gm.
in distilled water ..... 100 c.c.

Add 6 c.c. of this solution (B) to peptone solution (A) and boil.

C. **Ringer's Solution**

Dissolve:

Sodium chloride	2.4 gm.
Calcium chloride	0.06 gm.
Potassium chloride	0.06 gm.
Sodium bicarbonate	0.06 gm.
in distilled water	300 c.c.

Add 300 c.c. Ringer's solution to peptone solution (A and B) and boil.

D. **Sorenson's Buffer Solution**

1. M/15 KH_2PO_4

Dissolve:

Potassium phosphate (monobasic)	9.08 gm.
in distilled water	1000 c.c.

2. M/15 Na_2HPO_4

Dissolve:

Sodium phosphate (dibasic) anhydrous	9.47 gm.
in distilled water	1000 c.c.

Mix 75 c.c. of the monobasic potassium phosphate with 75 c.c. of the dibasic sodium phosphate. Add to the Peptone solution (A, B, and C). Boil until precipitation is complete (about 30 minutes). Cool in refrigerator overnight. Filter. Test pH which should be 6.8 to 7.2. Distribute in suitable containers. Autoclave at 15 pounds for 15 minutes. Test for sterility. Store in refrigerator until needed.

E. **Rabbit Serum**

To Verwoort's medium (A. B. C, and D) add 8 to 10 per cent sterile rabbit serum. Aseptically tube medium in 3 c.c. amounts. Inactivate in a 56° C. waterbath for 30 minutes. Test for sterility by incubating at 37.5° C. for 24 hours. If sterile, may be stored in refrigerator for several weeks until needed.

47. WAYSON'S STAIN

Dissolve:

Basic fuchsin (certified)	0.2 gm.
Methylene blue (certified)	0.75 gm.
in ethyl alcohol (absolute)	20 c.c.
When dissolved, add the dye solution to 5 per cent phenol in distilled water	200 c.c.
Filter	

Stain smears for a few seconds. Wash with water and drain and/or blot dry.

48. WRIGHT'S STAIN

A. **Stain**

Dissolve Wright's stain (certified) ..... .1 gm.
in methyl alcohol (absolute, acetone free, neutral) .....60 c.c.
Filter before using.

B. **Buffer Solution** (pH 6.4)

Dissolve Acid Potassium Phosphate (KH_2PO_4) .....6.63 gm.
Dibasic Sodium phosphate ($Na_2HPO_4.2H_2O$) ..... 3.2 gm.

Make to 1000 c.c. with distilled water and keep this solution in refrigerator as it is subject to mold contamination and decomposition in a few weeks at room temperature.

STAINING PROCEDURE—Cover blood smear with 15 drops of Wright's stain solution (A) and allow to remain for two minutes. Add an equal amount of distilled water or buffer solution (B) and mix by gently blowing on surface of stain-buffer mixture (stain should now have a metallic sheen). Allow diluted stain to remain for three more minutes. Wash with tap water and stand on end to dry.

NOTE:
If precipitated stain appears on slide it may be removed by rapidly flooding slide with staining solution (A) and immediately washing in running tap water.

49. ZIEHL NEELSEN'S CARBOLFUCHSIN (acid-fast) STAIN

Dissolve:

Basic fuchsin (certified)
(10 per cent alcoholic (95%) solution) ..... 10 c.c.
5 per cent phenol in distilled water .....100 c.c.

STAINING PROCEDURE

A. Flood the fixed smear with stain and steam gently over the flame for about 3 minutes (do not boil). Renew stain, as it evaporates. Allow to cool. Or

B. Place fixed smear in container of stain (coplin jar) and allow to set at room temperature for 12 to 24 hours.

After either A or B methods of staining wash with tap water and decolorize by dropping acid alcohol (2 per cent hydrochloric acid (USP) in alcohol (95%) over the smear until the alcohol flows colorless from the slide. (For B. leprae use 3 per cent nitric acid or 25 per cent muriatic acid). Wash with water. Counterstain with methylene blue for one minute. Wash with water, drain and/or blot dry and examine. Acid-fast bacteria stain red, others blue.

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